SEX STEROID HORMONE INFLUENCE ON ENDOCANNABINOID SYSTEM IN THE FEMALE REPRODUCTIVE TRACT

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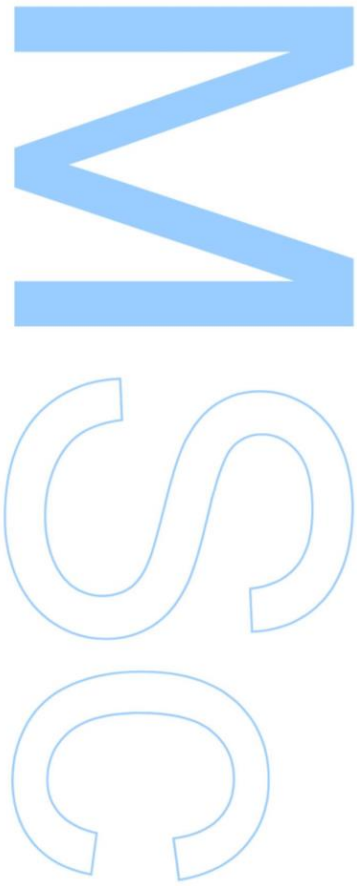

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Abstract

Anandamide (AEA), the major endocannabinoid (eCBs), is known to be involved in the modulation of several physiological functions, including endocrine regulation. In CNS, sex hormones modulate the expression of endocannabinoid system components and high AEA levels suppress the release of luteinizing hormone in ovariectomized (OVX) rats suggesting that changes in estradiol (E2) levels can influence cannabinoid signal in a region specific way. However, there are no evidences about the regulation of eCBs in uterus. The aim of this study is to elucidate whether the E2 administration to OVX rats can induce changes in the expression of cannabinoid receptors and AEA-metabolic enzymes in uterus, as well as, in plasmatic and uterine AEA, PGE_2 and $PGF_{2\alpha}$ levels. The effects of tamoxifen (TAM), an estrogen receptor modulator, were also determined. Besides uterine morphological analysis by Hematoxilin & Eosin and Masson's trichrome staining, it was performed western blot, RT-PCR, immunohistochemistry and LC-MS/MS. Present data show that estradiol benzoate (EB) administration to OVX rats significantly increases CB1, CB2, NAPE-PLD, FAAH and COX-2 expression which was confirmed with the qRT-PCR results. Moreover, this effects were absent with TAM treatment and also in TAM+EB treatment, which shows that this response is estrogen receptor dependent. The histological analysis also illustrates that the two receptors, as well as AEA-metabolic enzymes are located mainly in the epithelial cells of both lumen and glands and, to a lesser extent, in the muscle cells. Stereological analysis revealed that EB treatment led to a decrease in endometrium, a myometrium volume increase and also a higher number and volume of glands. AEA quantification showed no changes in its uterine levels in EB or TAM treated animals but showed a rise with EB treatment in plasma. EB also produced an increase in uterine PGE_2. In summary, these data collectively indicate that the expression of endocannabinoid system components, as well as, the AEA and PGE_2 levels in rat uterus is regulated by E2. Based in these results, along with the existing data, sex hormones, such as E2, may have a direct regulatory role in the modulation of endocannabinoid system in female reproductive tissues.

Key Words: Endocannabinoids; Estradiol; Anandamide; Prostaglandins; Tamoxifen

Resumo

A anandamida (AEA), principal endocanabinóide (eCBs), está envolvida na modulação de diversas funções fisiológicas, incluindo a regulação endócrina. No sistema nervoso central (CNS), as hormonas sexuais modulam a expressão dos componentes do sistema endocanabinóide bem como altos níveis de AEA suprimem a libertação de hormona luteinizante em ratos ovarietomizados (OVX) sugerindo que alterações dos níveis de estradiol (E2) podem influenciar especificamente a sinalização canabinóide. No entanto não existem estudos sobre a regulação dos eCBs no útero. Assim, este trabalho pretende estudar o efeito do E2 em ratos OVX relativamente a alterações na expressão dos recetores canabinóides e enzimas metabólicas da AEA, assim como, os níveis plasmáticos e uterinos de AEA, PGE_2 e $PGF_{2\alpha}$. É ainda pretendido compreender os efeitos do tamoxifeno (TAM), um modulador do recetor do estrogénio. Além do estudo morfológico do útero, após coloração de Hematoxilina & Eosina e tricrómio de Masson, foram ainda utilizadas técnicas de western blot, qRT-PCR, imunohistoquímica e LC-MS/MS. Os dados obtidos mostram que a administração de benzoato de estradiol (EB) a ratos OVX aumenta significativamente a expressão de CB1, CB2, NAPE-PLD, FAAH e COX-2. Este efeito não se verificou com a administração de TAM assim como no tratamento TAM+EB, o que mostra que esta resposta é dependente do receptor de estrogénio. A análise histológica revelou que tanto os receptores como as enzimas em estudo se localizam maioritariamente nas células epiteliais do lúmen e glândulas e, embora com menos expressão, também no miométrio. A análise estereológica revelou que no tratamento com EB ocorreu uma diminuição do volume relativo do endométrio e um aumento do volume do miométrio, assim como um aumento no número e volume das glândulas. A quantificação de AEA não revelou alterações nos diferentes tratamentos no útero, mas a nível plasmático observou-se um aumento em ratos tratados com EB. O tratamento com EB produziu ainda um aumento de PGE_2 uterino. Em suma, os resultados obtidos indicam que a expressão dos vários elementos do sistema endocanabinóide, bem como os níveis de AEA e PGE_2 são reguladas pelo E2. Com base neste estudo assim como na literatura existente, as hormonas sexuais, como o E2, podem ter um efeito regulatório direto na modulação do sistema endocanabinóide nos tecidos reprodutores femininos.

Palavras-chave: Endocanabinóides; Estradiol; Anandamida; Prostaglandinas; Tamoxifeno

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List of abbreviations

AA-arachidonic acid

AC-adenylyl cyclase

ACN-acetonitrile

2-AG-2-arachidonoylglycerol

AEA-Anandamide

AF1-activation function 1

AF2-activation function 2

CAST-Computer Assisted Stereology Toolbox

CB1-cannabinoid receptor 1

CB2-cannabinoid receptor 2

CNS-central nervous system

COX-1-cyclooxygenase-1

COX-2-cyclooxygenase-2

DAG-diacylglycerol

DAGL-diacylglycerol lipase

E2-estradiol

EB-estradiol benzoate

eCBs-endocannabinoids

ECS-endocannabinoid system

EMT-endocannabinoid membrane transporter

ER-estrogen receptor

ERα-estrogen receptor α

ERβ-estrogen receptor β

ERE-estrogen response elements

ERK-extracellular signal-regulated kinase

FAAH-fatty acid amide hydrolase

FAK-focal adhesion kinase

FAN-neutral sphingomyelinase activation

FDA-Food and Drug Administration

FSH-follicle stimulating hormone

GASP1-Gprotein receptor-associated sorting protein 1

GPCRs-G-protein coupled receptor

HE-hematoxylin & eosin

IVF-*in vitro* fertilization

JNK-c-jun N-terminal kinase

LOXs-lipoxygenases

LPI-lysophosphatidyinositol

MAGL-monoacylglycerol lípase

MAPK-mitogen-activated protein kinase

MeOH-methanol

NAPE-*N*-arachidonoyl-phosphatidylethanolamine

NAPE-PLD-NAPE phospholipase D

NAT-*N*-acyltransferase

OVX-ovariectomized

PAGE-polyacrylamide

PBS-phosphate buffer

PCR-polymerase chain reaction

PG-EA-prostaglandin-ethanolamides

PGs-prostaglandins

PGE2-prostaglandin E2

PGES-Prostaglandin E synthase

$PGF_{2\alpha}$-prostaglandin F2α

PGH_2-prostaglandin H2

PKA-protein kinase A

PKC-protein kinase C

PLC-phospholipase C

PPARs-peroxisome proliferator-activated receptors

PTX-pertussis toxin

SEM-standard error of the mean

SERM-selective estrogen receptor modulator

TAM-tamoxifen

THC-Δ^9-tetrahidrocanabinol

TRPV1-transient receptor potential vanilloid 1

Chapter I

Introduction

Introduction

1. Cannabinoids: Historical background

Cannabis sativa has been used with medical purposes as well as a recreational drug for hundreds of years. Nowadays, its derivatives are the most widely used illicit drugs in several countries throughout the world. Of all the active compounds of the plant, almost 60 belong to the cannabinoid class, which is composed by the chemical molecules that can activate the cannabinoid receptors, being Δ^9-tetrahydrocannabinol (THC) the main psychoactive component [1] (Fig.1).

Nowadays, after years of investigation, more compounds of the cannabinoid class have been discovered and produced in a synthetic way, with some of them being actually used in the clinic.

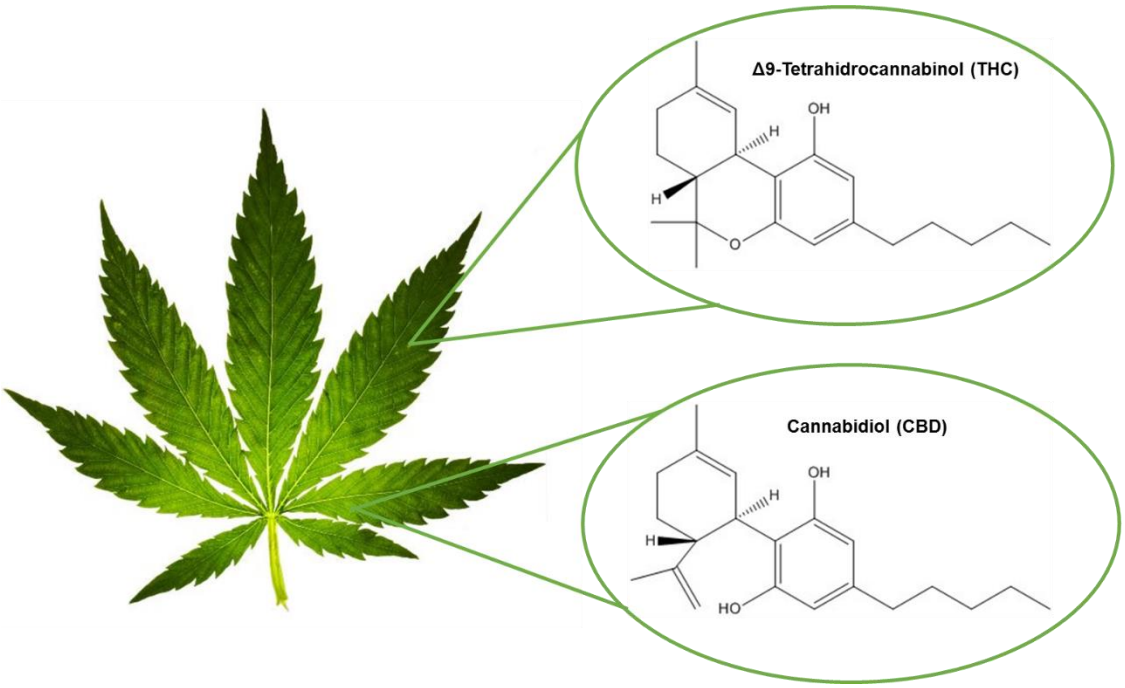

Figure 1. *Cannabis sativa* and its main active components. The chemical structure of THC and CBD is depicted.

1.1 Cannabinoid Receptors

In 1990, the structure of the first cannabinoid receptor along with its functional expression of cloned cDNA was achieved [2]. This receptor was later named cannabinoid receptor 1 (CB1) and is present mainly in the central nervous system (CNS) but also, in

less extent, in peripheral tissues such as the immune system, endothelium tissue, intestine, liver, peripheral nerve synapses and reproductive tissues [3].

Three years after CB1 discovery, a second cannabinoid receptor was found in the periphery, the cannabinoid receptor 2 (CB2) [4]. Contrary to CB1, it exists mainly in immune cells such as B cells and natural killer, even though both receptors can be co-located in some tissues or organs [3] (Fig. 2). Besides these two receptors, there is also a possible third cannabinoid receptor named orphan G-protein coupled receptor 55 (GPR55), which was discovered during *in silico* patent searching and is predominantly located in the human brain and liver [5].

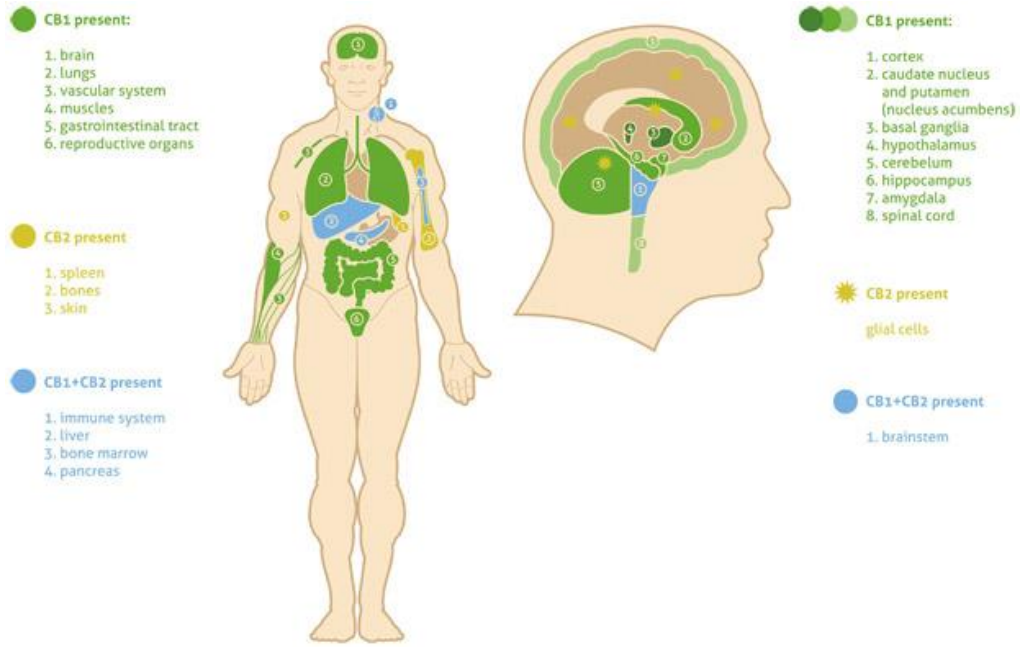

Figure 2. Localization of cannabinoid receptors in the human body. Although both cannabinoid receptors are widely distributed in the body, CB1 is primarily expressed in the brain, whereas CB2 receptor predominates in immune cells. (Adapted from "*fundación canna*").

1.2 Endocannabinoids

The characterization of cannabinoid receptors led to the idea that endocannabinoid-like compounds could exist in our body. In 1992, the first endogenous ligand for these receptors was identified and named anandamide (AEA) [6]. This ligand was found to have similar effects to the natural and synthetic cannabinoids such as analgesia, motor depression, catalepsy as well as hypothermia [7].

Three years later, a second endogenous ligand for cannabinoid receptors was discovered and named 2-arachidonoylglycerol (2-AG) [8]. More endogenous compounds

such as amides, esters or ethers of long-chain polyunsaturated fatty acids may also bind to the cannabinoid receptors and are suggested as endocannabinoids (eCBs) such as virodhamine, noladin ether, N-arachidonoyldopamine, N-arachidonoylglycine and oleamide [3] (Fig. 3).

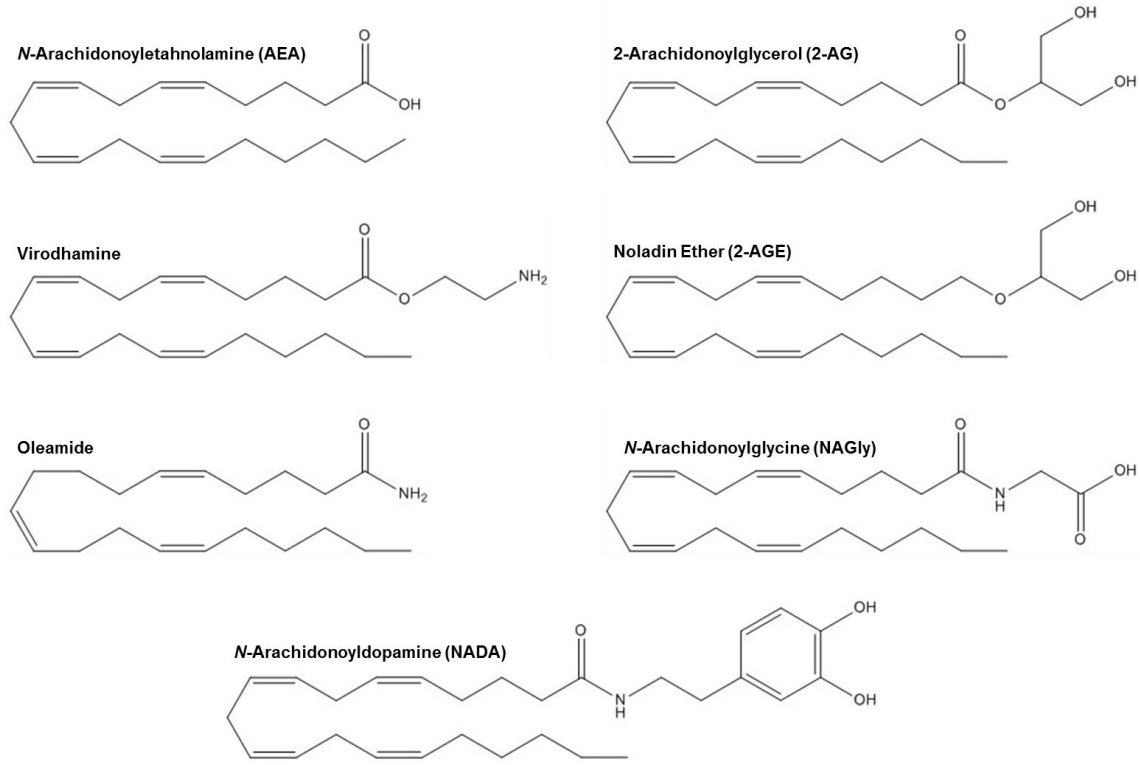

Figure 3. Chemical structure of the main endocannabinoids and cabamimetics. Endogenous cannabinoids Anandamide (AEA) and 2-Arachidonoylglycerol (2-AG) along with Virodhamine, Noladin Ether (2-AGE), Oleamide, N-Arachidonoylglycine (NAGly) and N-Arachidonoyldopamine (NADA), the main cabamimetics capable of cannabinoid receptor binding.

1.3 Synthesis and Degradation

Unlike the other classical neurotransmitters, eCBs are not stored in cells but instead, are produced mainly “on demand” by cleavage of membrane phospholipids precursors. As any molecule acting as a mediator of physiological or pathological responses, the synthesis and degradation of the eCBs has to be tightly regulated.

AEA is produced by the cleavage of a phospholipid precursor, the N- arachidonoyl-phosphatidylethanolamine (NAPE), which is synthesized by N-acyltransferase (NAT), by transferring arachidonic acid (AA) from phosphatidylcholine to the head group of phosphatidylethanolamine. It occurs through enhanced intracellular Ca^{2+} concentrations, e.g. from cell depolarization or mobilization of intracellular Ca^{2+} stores. Thus, the release of AEA from NAPE occurs by a specific phospholipase D (NAPE-PLD) [9] found in the

inner layer of the cell membrane. Besides the brain, where this enzyme is found in high levels, it can also be found in kidney, spleen, lung, heart, liver and uterus [3].

2-AG has a different metabolic pathway than AEA. The first step of its synthesis is the hydrolysis of membrane phospholipids by phospholipase C (PLC), producing 1,2-diacylglycerol (DAG), which is then converted to 2-AG by diacylglycerol lipase (DAGL) [10].

After biosynthesis and release to the extracellular space to activate its receptors, cessation of cannabinoid signal occurs in a two-step process, which includes the transport from the extracellular space to the intracellular space and degradation by hydrolysis or oxidation. Due to their lipophilic character, the eCBs can also diffuse through the plasma membrane. Intracellular sequestration of eCBs by binding to intracellular proteins has been suggested as contributing for eCBs uptake but only recently, these proteins were identified as Hsp70, FABP5 and FABP7 [11] [12, 13]. The facilitated diffusion driven by a carrier-protein has also been described and partially characterized. The endocannabinoid membrane transporter (EMT) mediates bidirectional transport [14].

On the other hand, the intracellular levels of eCBs are dictated by the degradative enzymes. The AEA degradation is primarily mediated by fatty acid amide hydrolase (FAAH) [15]. The continuous degradation by FAAH creates and maintains the necessary gradient to drive the process of simple diffusion [16] (Fig.4). As AEA presents structural similarities with other polyunsaturated fatty acids, it can also be degraded by the inducible cyclooxygenase-2 (COX-2) as well as by various lipoxygenases (LOXs) in an analogous manner to that seen for AA. In fact, AEA serves as substrate for COX-2, but not for cyclooxygenase-1 (COX-1), resulting mainly prostaglandin-ethanolamides (PG-EA), also termed prostamides [17, 18].

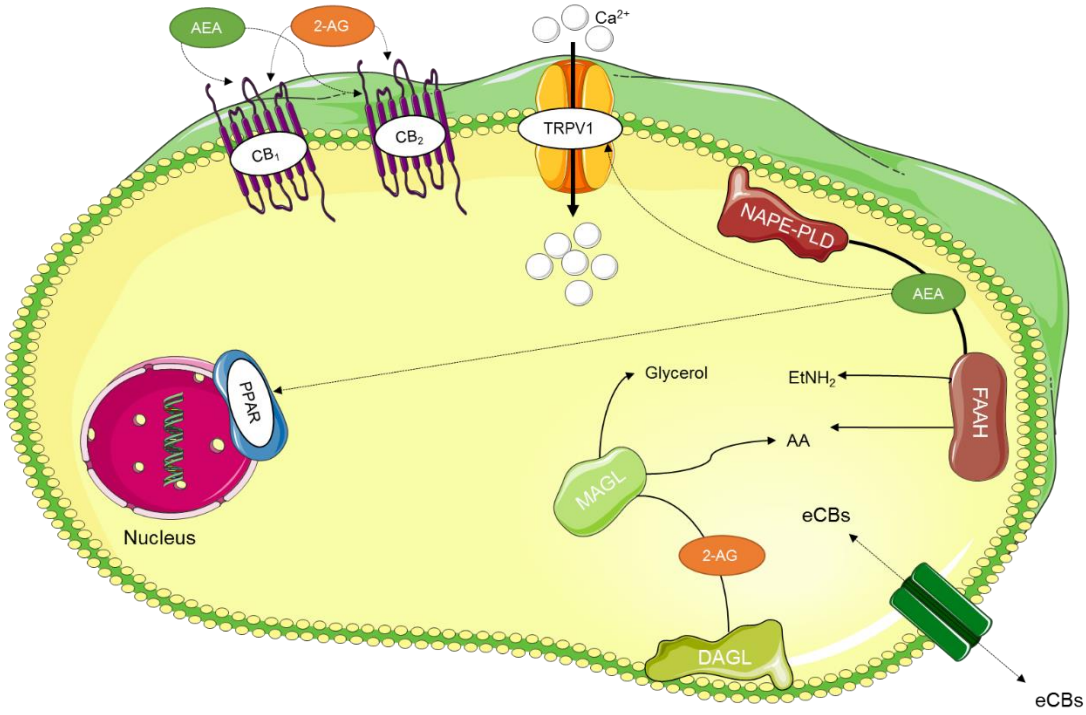

Figure 4. Biochemistry of the endocannabinoid system. Enzymes responsible for AEA and 2-AG synthesis and degradation, the main receptors that can be activated by this eCBs and their membrane transporter.

1.4 Endocannabinoid Signaling

The CB1 receptor is the most abundant G-protein coupled receptor (GPCR), with densities 10–50-fold above those of classical transmitters, such as dopamine or opioid receptors in the brain [3].

Cannabinoid effects are affected by pertussis toxin (PTX), indicating that cannabinoid receptors bind to a $G_{i/o}$ protein. There are also evidences that CB1 receptors can interact with G_s under PTX treatment conditions that prevents receptors interaction with $G_{i/o}$ proteins [19].

CB1 stimulation modulates adenylyl cyclase (AC) and, consequently, intracellular cAMP levels [20], thereby regulating protein kinase A (PKA) phosphorylation resulting in major changes in cellular activity [20]. On the other hand, it is established that interaction with Gi/o protein activates different kinases, such as p38 mitogen-activated protein kinase (MAPK) [21], focal adhesion kinase (FAK) [22], c-jun N-terminal kinase (JNK) [23] and extracellular signal-regulated kinase (ERK) [24] (Fig. 5). Moreover, the CB1 receptor may directly inhibits N- or P/Q-type Ca^{2+} channels and activates inwardly rectifying K^+ channels [25].

In addition to these well-established events, recent developments have shown that the signaling pathways induced by cannabinoid receptors are more diverse than

originally recognized. In fact, new observations revealed that cannabinoids can modulate sphingolipid-metabolizing pathways by increasing the intracellular levels of ceramide [26], an ubiquitous lipid secondary messenger that can control cell fate. In fact, CB1 receptor activation has been shown to induce sphingomyelin hydrolysis and acute ceramide production within minutes in both primary astrocytes [27] and C6 glioma cells [28], through sphingomyelinases. Different adaptor proteins are involved in the functional coupling of CB1 with sphingomyelinases, one of which is the factor associated with neutral sphingomyelinase activation (FAN). Actually, it was shown that cells expressing dominant-negative FAN are resistant to cannabinoid-induced sphingomyelin breakdown [29]. Nonetheless, activation of CB1 also induces an extended ceramide accumulation due to the stimulation of its de novo synthesis by serine palmitoyltransferase [26].

Furthermore, recently it was observed that some proteins interact specifically with CB1 receptors and interfere with its signaling, such as the CB1 receptor interacting proteins, CRIP1a and CRIP1b [30], G protein receptor-associated sorting protein 1 (GASP1) [31] and β-arrestins [32].

CB2 receptor activation does not modulate ion channel function, as demonstrated in CB2 transfected AtT-20 cells and Xenopus oocytes [33], or couple to Gs protein [19]. On the other hand, CB2 stimulation modulates MAPK pathway as well as nitric oxide production [34].

The G-protein coupling associated with GPR55, the purported CB3, seems to be more complex and is still largely undefined, even though there were some agonists found such as lysophosphatidyinositol (LPI) derivates, which result in robust stimulation of the receptor [35]. In spite of that, it is well accepted that GPR55 promotes ERK phosphorylation and increases intracellular Ca^{2+}. Several other pathways are associated with GPR55 activation depending on type and ligand concentration [36].

In addition to the activities through cannabinoid receptor activation, AEA may also interact with other receptors such as the transient receptor potential vannilloid 1 (TRPV1) [37] and the peroxisome proliferator activated receptors (PPARs) family [38]. TRPV1 phosphorylation by protein kinase C (PKC) and PKA sensitises TRPV1 receptors to vanilloids, AEA, heat and protons. Repeated administration of AEA gradually evokes larger TRPV1 currents in oocytes and enhances proton-evoked currents. The potentiation by AEA is significantly reduced by compounds that inhibit PKC, suggesting that AEA stimulates PKC to enhance agonist-evoked responses [39].

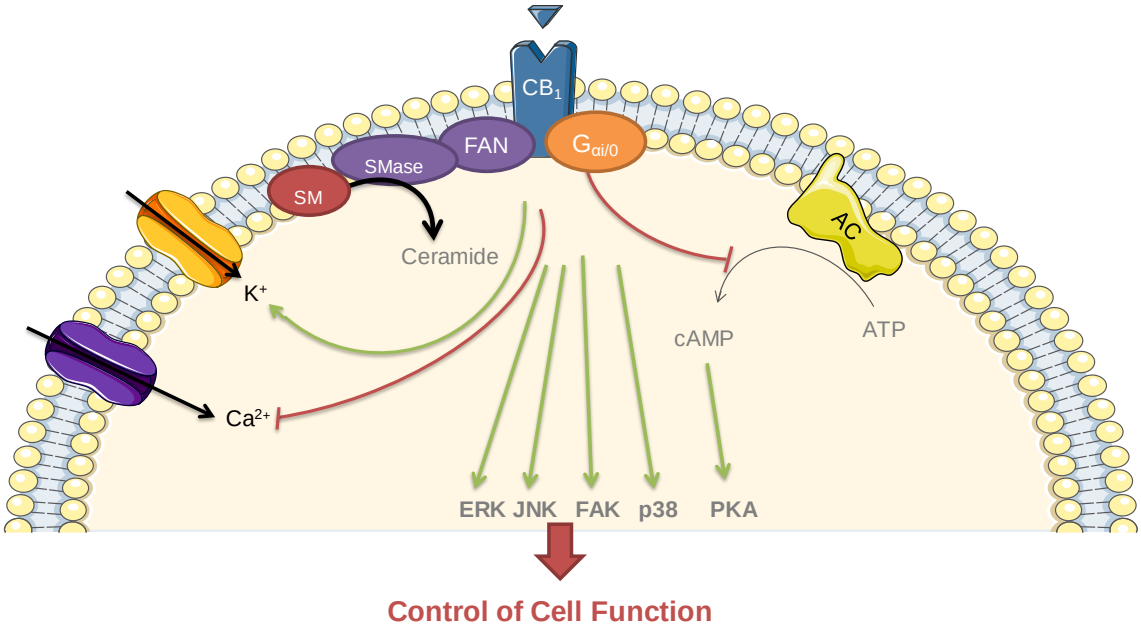

Figure 5. Cannabinoid receptor signaling. Cannabinoids exert their effects by binding to specific G-protein-coupled receptors. The major pathways and channels activated by CB1 receptor are depicted. These include inhibition of the adenylyl cyclase (AC)–cyclic AMP–protein kinase A (PKA) pathway; inhibition of voltage-operated Ca^{2+} channels and activation of K^+ inwardly rectifying channels; activation of mitogen-activated protein kinase cascades (ERK; JNK, p38 and FAK); and ceramide generation.

AEA can activate PPARs through different pathways: it can interact directly with the receptor [40]; indirectly by its metabolites, as has been shown by the lack of PPAR activation when COX-2 is inhibited [41]; and also by cannabinoid receptor activation at the cell surface, which initiates intracellular signaling like ERK-1/2 or p38 that may lead to PPAR activation [38].

1.5 Phisiological Function

Endocannabinoids can influence a wide range of physiological activities, including nociception, cardiovascular, respiratory and gastrointestinal (GI) function.

In the brain, there are several lines of evidence that cannabinoid system activity modulates critical molecular and cellular processes influencing the pace of the ageing process. Phytocannabinoids and structurally related synthetic compounds are known to possess anti-oxidant properties. The protective effect of cannabinoids against oxidative stress in vitro in neurons is dependent on their anti-oxidant properties as cannabinoid receptors are not involved in this process [42, 43]. However, in astrocytes, the CB1 receptor mediates the protective effect of cannabinoids. For the neuroprotective effect of cannabinoids both their anti-oxidant and receptor binding properties contribute, as was shown in a rodent model of Parkinson's disease [44]. Besides its anti-oxidant properties,

cannabinoids can also improve the clearance of damaged macromolecules, at physiological concentrations, improving lysosomal stability and integrity, which may be helpful against Alzheimer disease [45].

Mouse models lacking CB receptors give support to significant and balanced roles of endocannabinoid signaling in male and female fertility events such as spermatogenesis, fertilisation, preimplantation development of the early embryo, implantation and post implantation growth of the embryo [46].

Interactions of endocannabinoids with hypothalamic hormones are believed to influence food intake. Activation of the endocannabinoid system (ECS) promotes consumption of palatable food, stimulates fat mass expansion and calorie preservation, while inhibiting energy expenditure and thermogenesis. However, in the modern society where food is plentiful, excessive ECS activity is a landmark feature of obesity and metabolic disorders [47, 48]. This interaction between food intake and ECS culminated in the discovery of Rimonabant, an anorectic drug and systemic CB1R inverse agonist, which was approved for anti-obesity therapy in Europe, but by the end of 2008 it was withdrawn because of its psychiatric side effects [49]. However new drugs, some of them CB1 antagonists, are being developed with the intention of reducing body weight as well as food intake [50]. Besides food intake, cannabinoid receptors may also play a role in nausea and emesis, gastric secretion and gastroprotection, gastrointestinal motility, ion transport, visceral sensation, intestinal inflammation, and cell proliferation in the gut [51].

Cannabinoids also have immunosuppressive properties. They can inhibit proliferation of leucocytes, induce apoptosis of T cells and macrophages and reduce secretion of pro-inflammatory cytokines [52]. In mice models, they are effective in reducing inflammation in arthritis, multiple sclerosis, have a positive effect on neuropathic pain and in type 1 diabetes mellitus. Studies in human models are insufficient and not conclusive and more research is required in this field. Cannabinoids can be therefore promising immunosuppressive and anti-fibrotic agents in the therapy of autoimmune disorders [52].

ECS is also present in both anterior and posterior ocular tissues including the retina [53]. The presence of these components supports an important role for the ocular ECS in the endogenous signaling of both the anterior and posterior eye. Application of cannabinoids to the eye produces a variety of effects, notably hyperemia, reduced tear production, and a reduction in intraocular pressure [54]. Of these effects, the intraocular pressure lowering properties of cannabinoids have attracted considerable attention with respect to the possibility of developing cannabinoid-based therapeutics for glaucoma.

Many compounds that either act directly at the receptor or increase/reduce ligand availability have the potential to affect other brain functions and cause side effects. Novel

drug targets such as FAAH and MAGL inhibitors appear to be promising in animal models, but more studies are necessary to prove their efficiency.

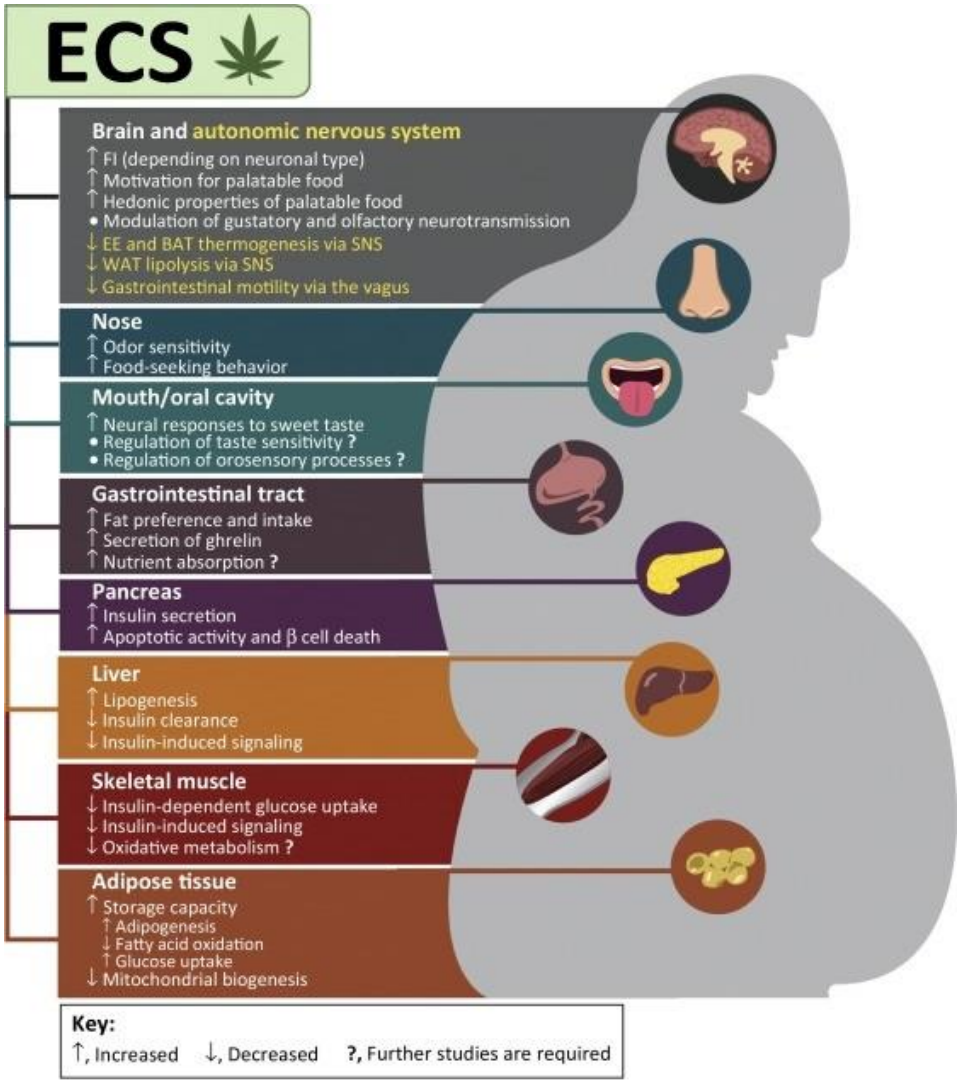

Figure 6. Main physiological functions affected by ECS. The main functions attributed to ECS are in the brain and autonomic nervous system, nose and oral cavity, gastrointestinal tract, pancreas, liver, muscle and adipose tissue. (Adapted from Mazier et al, 2015 [49])

2. eCBs, Estradiol and PGs cross-talk in reproduction

Endocannabinoids affect reproductive events from gametogenesis to fertilization and embryo implantation to the final outcome of pregnancy. Thus, they have been proposed as suitable biomarkers to predict the reproductive potential of male and female gametes in clinical practice [55]. The physiological tone of AEA and 2-AG is critical to preimplantation events in mice, since silencing or amplification of these signaling pathways cause underdevelopment and oviductal retention of embryos via CB1, leading

to deferred implantation and compromised pregnancy outcome [56]. Moreover, low AEA levels have been detected during the implantation window in women with successful pregnancy after *in vitro* fertilization treatment [57].

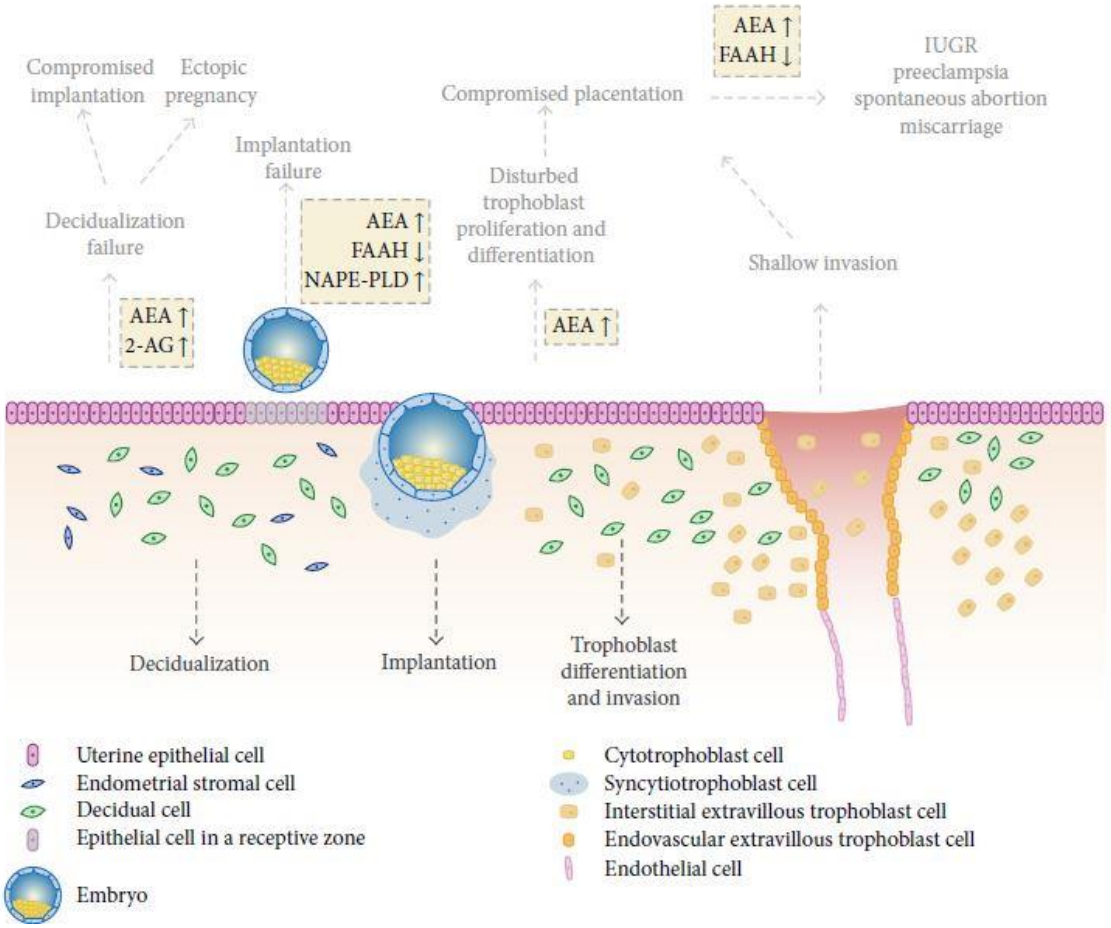

Figure 7. Schematic representation of the fetomaternal interface. Deregulated endocannabinoid signaling in the fetomaternal interface may compromise implantation and placentation and result in ectopic pregnancy, intra uterine growth restriction (IUGR), preeclampsia, spontaneous abortion and miscarriage (Adapted from Fonseca et al, 2013 [58]).

Elements of the endocannabinoid system like CB1 receptor are expressed in the embryo in much higher levels than those in the brain [59]. AEA was also found in much higher levels in the mice non pregnant uterus than in brain, which, together with the changing levels of AEA with pregnancy status, is indicative of a role for this lipid in early pregnancy events [60].

Low endocannabinoid levels allow the appropriate environment conducive to preimplantation embryo transport through the oviduct [61]. In fact, there is a regional regulation with higher expression of FAAH and NAPE-PLD in its ampulla and isthmus, respectively. This differential expression creates the appropriate AEA levels during oviductal transport (Fig. 7).

A similar phenomenon is observed in mice uterus during implantation where the expression of AEA-metabolic enzymes in mouse uterus is critical to define their concentration in implantation sites and, consequently, in the implantation outcome. It has been shown that the expression of placental CB1 receptor is elevated, along with decreased FAAH, in spontaneous miscarriage in the first trimester compared with levels observed in voluntary termination [62]. In fact, just before embryo implantation, AEA declines to barely detectable levels at the site of implantation, and this change is believed to contribute to the receptive uterine state [60]. AEA can also induce differential signals in blastocyst differentiation and outgrowth. At low levels, cultured blastocysts exhibited accelerated trophoblast differentiation and outgrowth, while higher levels induce opposite effects [63, 64].

Furthermore, eCBs and a synthetic cannabinoid (CP55, 940) stimulate fetal membrane production of PGE_2 in a CB1 receptor-dependent manner [65]. Similar results were also obtained using rat uterus, although in this tissue AEA increased the levels of PGE_2 and $PGF_{2\alpha}$ in a CB2 receptor-dependent manner [66].

It has been described that COX-2 mediates several cellular events of eCBs, and that AEA modulates PGs production and COX-2 expression in reproductive tissues and other systems [67]. Therefore, endocannabinoids have labour-promoting effects via PG production (Fig. 8).

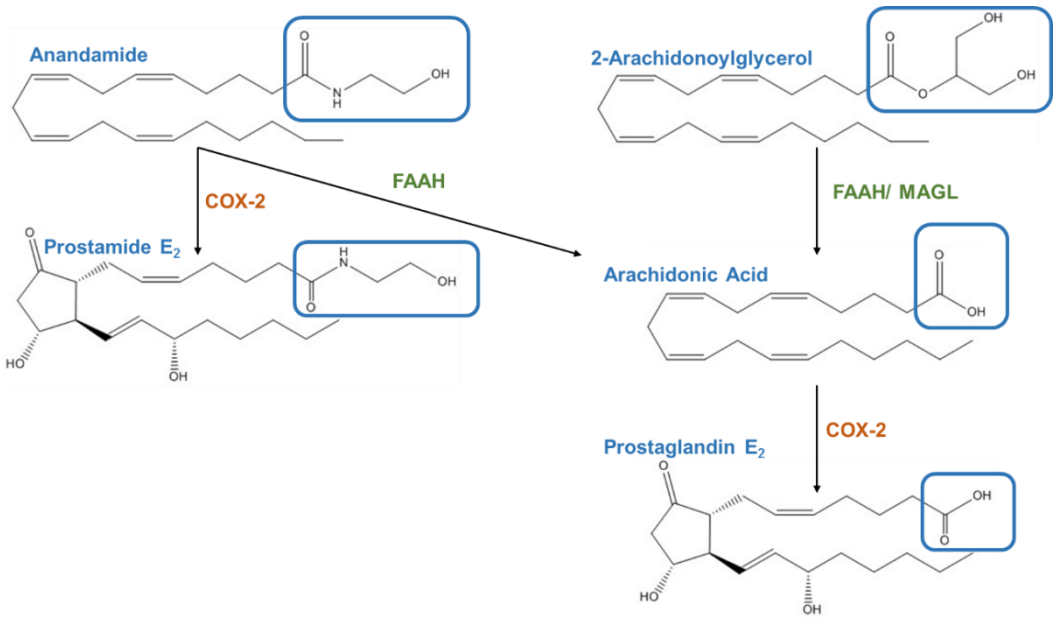

Figure 8. Structural relationship between PGs and ECS. AEA and 2-AG degradation results in the AA production which is used for prostaglandin production. AEA can also be metabolized by COX-2 into prostamides.

Steroids, as endogenous hormones, may interact with the fertilised ovum and modulate PGs levels. Although poorly studied, these interactions can also dictate the

outcome of fertilization and pregnancy progression. Previous studies have shown that estradiol (E2) administration to ovariectomized (OVX) rats led to an increase in $PGF_{2\alpha}$ but also a decrease in PGE_2, and this effects were blocked by progesterone [68]. This decrease in PGE_2 production can be explained by a decrease in mRNA levels of PGES by E2 when administrated to ovariectomized ewes [69]. Also, in this study PGES activity decrease by E2 was blocked by progesterone. Even though there is some information about sex steroid hormones influence on PGs production, this information is a little outdated. With more updated instrumentation, this information can be confirmed.

Prostaglandins (PGs) play a critical role in the mechanisms of miscarriage [70] and parturition at both term and preterm [71]. Cyclooxygenase is the rate-limiting enzyme in prostaglandin (PG) biosynthesis and exists in two isoforms, COX-1 and COX-2. These enzymes catalyse the conversion of AA into prostaglandin G2, which are further peroxidised to prostaglandins H_2 (PGH_2) [72]. Prostaglandin E synthase (PGES) is a terminal prostanoid synthase and can enzymatically convert the cyclooxygenase product PGH_2 to prostaglandin E2 (PGE_2), which can further bind to and activate a set of functionally distinct cell surface receptors named prostaglandin E2 receptors (EP) - EP1, EP2, EP3 and EP4 [73]. COX-1 is known to be a constitutive enzyme expressed in most tissues [74], whereas expression of COX-2 can be induced by cytokines/growth factors or inflammatory stimuli.

COX-2 is important during ovulation, fertilization, implantation and decidualization as showed in COX-2 $^{-/-}$ mice, which presented compromised implantation and decidualization [75]. PGE_2 is suspected to be implicated in the increase of vascular permeability during implantation and is known to be essential for decidualization [70]. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the key luteolytic factor in the female reproductive tract of most mammals [76]. In fact, $PGF_{2\alpha}$ has also been considered as the primary candidate present during pregnancy, where it plays a crucial role in myometrium during parturition by increasing the oxytocin-induced contractions [77].

The panoply of molecules that contributes to uterine microenvironment is complex, and, although isolated, these molecules were shown to be relevant for endometrial remodelling and pregnancy establishment and progression. The cross-talk between E2, PGs and eCBs is largely unexplored.

3. Ovarian steroid hormones

Sex steroids, estrogens and progesterone, are involved in several physiological mechanisms such as the menstrual cycle and pregnancy. The human menstrual cycle

begins with menstruation, which lasts for 3-6 days, during which the superficial layer of the uterine endometrium is shed. The endometrium regenerates during the follicular phase of the cycle after menstrual flow has stopped. Estrogens are responsible for the proliferative phase of endometrial regeneration, which occurs from day 5 or 6 until mid-cycle. This rise in estrogen level is accompanied by a drop in follicle stimulating hormone (FSH) level. Ovulation occurs 40–44 h after a luteinizing hormone surge and a milder FSH surge. The luteal phase is characterized by moderate levels of estrogens and increasing progesterone production by the corpus luteum. Estrogen decreases from moderate level at the midluteal phase to its lowest level just before the onset of menstruation. Progesterone levels rise after ovulation, peak approximately 7 days post ovulation and fall rapidly just before menstruation to undetected levels (Fig. 9) [78]. Progesterone acts on estrogen-primed endometrium, stimulating the secretory phase of the cycle, which renders the endometrium suitable for the implantation of a fertilized ovum. If implantation of a fertilized ovum does not occur, progesterone secretion stops, triggering menstruation. If implantation does occur the corpus luteum continues to secrete progesterone, which, by its effect on the hypothalamus and anterior pituitary, prevents further ovulation [79].

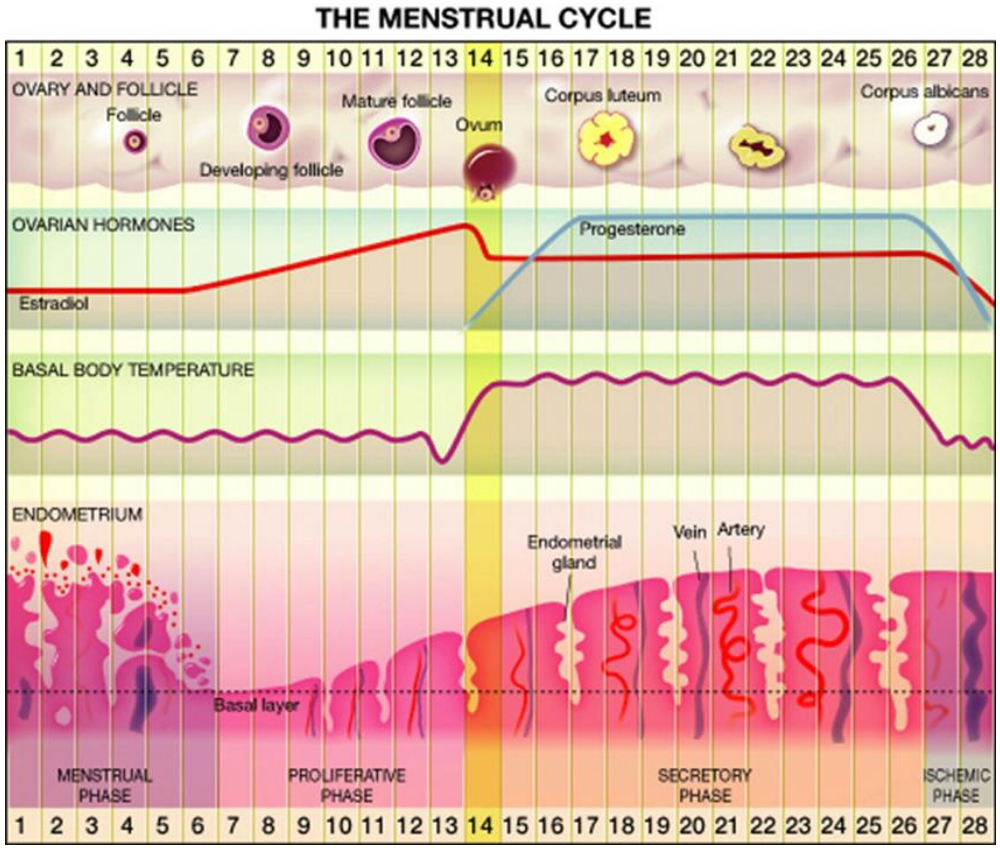

Figure 9. The menstrual cycle. Fluctuations of E2 and progesterone levels and the endometrial variations through the menstrual cycle (Adapted from Haimov-Kochman et al, 2014 [80]).

3.1. Estrogens

There are three naturally occurring estrogens: E2, estrone and estriol, all C18 steroids derived from cholesterol [81] (Fig. 10). E2 is mainly produced in the ovarian tissue, however there are many tissues with the ability to synthesize estrogens [82]. Upon the binding to lipoprotein receptors, cholesterol is uptaken by steroidogenic cells, stored, and moved to the sites of steroid synthesis. Different steroid hormones are produced by reduction of the number of carbon atoms from 27 to 18 [81]. The last step of estrogen production is the aromatization of androstenedione and testosterone, which are catalyzed by P450 aromatase monooxygenase enzyme complex [81]. This complex is found in various human tissues being some of them the ovarian granulosa cells, placental syncytiotrophoblast, adipose and skin fibroblasts, bone and brain [82].

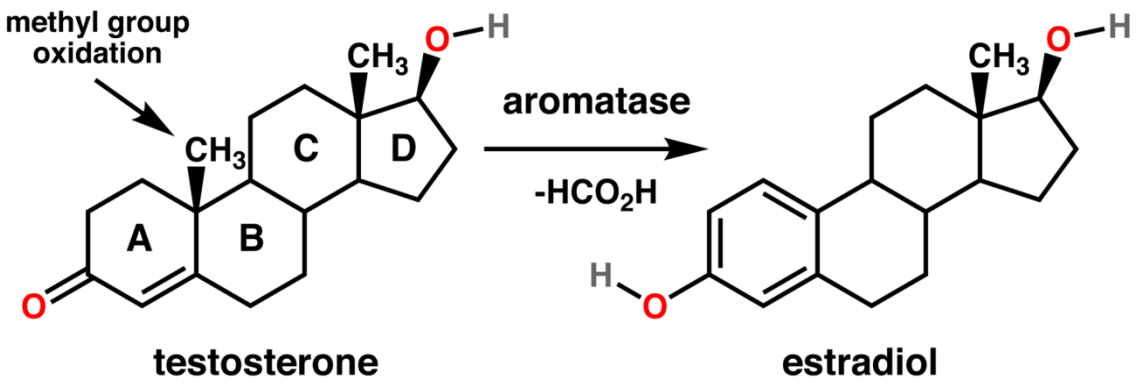

Figure 10. Aromatase complex reaction and E2 production. Methyl group is oxidated and removes by aromatase.

3.2. Rat uterus and hormone cycle

Rodents are spontaneous ovulators which means that, contrary to the induced ovulators, they do not need the presence of males to induce ovulation, although there is some evidence that male rodents can induce and/or hasten ovulation [83]. Rats and mice typically have a 4-5 day cycle which consists of 4 stages – estrus, proestrus, diestrus I (or metestrus) and diestrus II [84] (Fig. 11).

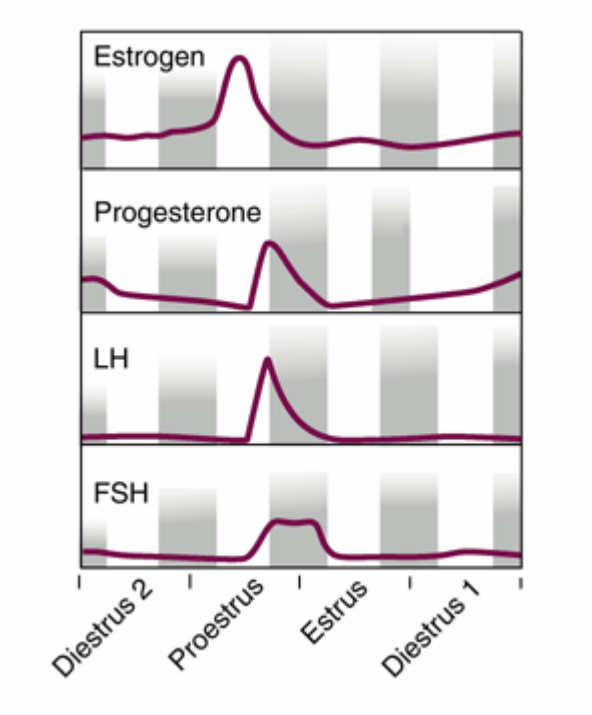

Figure 11. Sexual hormone variations during rat hormone cycle. Contrary to humans, different uterine hormones peak at the closer time during proestrus (Adapted from Staley et al, 2005 [85]).

In addition to the total time required for a complete cycle, one of the main differences between the cycle of a rodent and human is that E2 and progesterone in the rodent cycle is closer in time [84].

The fluctuation in hormone levels during the cycle results in changes in the number and types of cells present in the vagina. This is mostly due to the cyclical hypertrophy and subsequent sloughing of the uterine epithelium and also the concomitant invasion of white blood cells that scavenge the dead and dying cells [86]. Although cell density is not usually an accurate way to determine cycle stage, the cell type is. Protestrus is defined by the presence of nucleated epithelial cells that are round. Estrus is characterized by non-nucleated, cornified epithelial cells. Metestrus (or Diestrus I) contains mostly lymphocytes. Diestrus II typically has a low cell number, often with a lot of cell debris [86]. To determine the influence of estrogens such as E2, different models are used in which estrogen-replaced animals are compared with control groups where estrogen effects are attenuated [87]. The methods for estrogen removal or inhibition include ovariectomy and treatment with selective estrogen-receptor modulators (SERMs) [87]. Although ovariectomy leads to estrogen production by peripheral tissues along with tissue mass increase by the induced hyperphagia, the reduction of estrogen production through this method is the most common [88]. Moreover, these effects may be reduced by using an aromatase inhibitor or through diet control [87]. Besides

ovariectomy, E2 levels and its influence can be eliminated using knockout mice for aromatase. However, this method is more indicated for the study of E2 influence in the rodent development [89].

3.3. Phisiological actions

Estrogens main targets are the reproductive organs, but they also act on other organ systems such as cardiovascular, skeletal, immune, gastrointestinal, and neural sites [90-93]. Their major actions are genomic, mediated by nuclear estrogen receptors (ERs), but they also have non-genomic actions. There are two ERs (α and β) and both belong to the steroid hormone superfamily of nuclear receptors, which act as transcription factors. The ER-gene expression regulation by ERs modulate biological functions, such as reproductive organ development, bone remodeling, cardiovascular system functioning, metabolism, and behavior in both females and males [94]. These receptors have 5 domains from A to F listed from N- to C-terminal and in the N-terminal of the A/B domains of ERs consist of activation function 1 (AF1), which contributes to the transcriptional activity of ERs and is an essential domain for interaction with co-regulators. The E/F domain, located in the C-terminal region, contains a ligand-binding domain that serves as an interaction site with co-regulators and ligand-dependent activation function 2 (AF2). AF1 and AF2 control the transcriptional regulatory activity of ERs because activation of ERs are stimulated during cellular responses to the environment [94]. The general physiological roles of ERs has been understood using estrogen levels ablation by ovariectomy and then replenished by treatment with exogenous E2 [95].

3.4. Tamoxifen

Tamoxifen (TAM) is a first-generation selective estrogen receptor modulator (SERM) approved by the US Food and Drug Administration (FDA) for primary chemotherapy and adjuvant treatment of women with breast cancer [96] (Fig. 12).

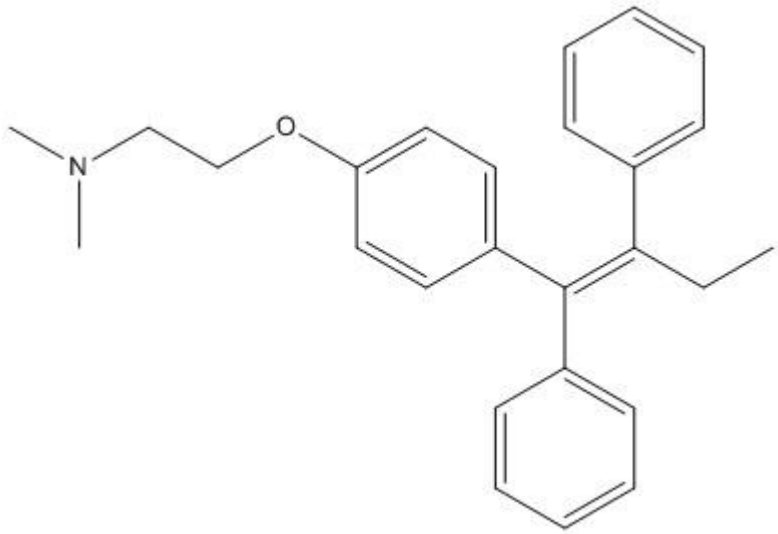

Figure 12. Tamoxifen chemical structure. Chemically tamoxifen is very similar to estrogen/E2 however E2 is a small carbon-rich steroid and tamoxifen has an extra chain, which is important for its antagonistic action.

Although TAM has been shown to be effective in the treatment of breast cancer, some tumors do not respond and develop resistance. There are many possible mechanisms to explain this resistance including the down-regulation, mutation, or loss of ERs and impaired co-activator signaling [97]. The anti-tumor effect of TAM is thought to be mediated by its anti-estrogenic effects. In a target cell, estrogen binds to ER, initiating a sequence of events (Fig. 13) [98]. The estrogen–ER complex homodimerizes and binds to discrete DNA sequences, known as estrogen response elements (ERE), in the regulatory regions of estrogen-sensitive genes. The two transcriptional activation functions of ER complex, AF1 and AF2, interact with other proteins (transcriptional coactivators) to stimulate the activity of RNA polymerase II, thereby regulating gene activity (Fig. 13A). TAM competitively inhibits the binding of estrogen to ER [99] and its complex with ER homodimerizes and binds to the ERE of estrogen sensitive genes (Fig. 13B) . However, TAM is only able to activate the AF1. The inactivity of AF2 results in an attenuation of transcription of the estrogen-responsive gene and of coactivator binding. As a result, TAM blocks the G1 phase of the cell cycle, slowing cell proliferation.

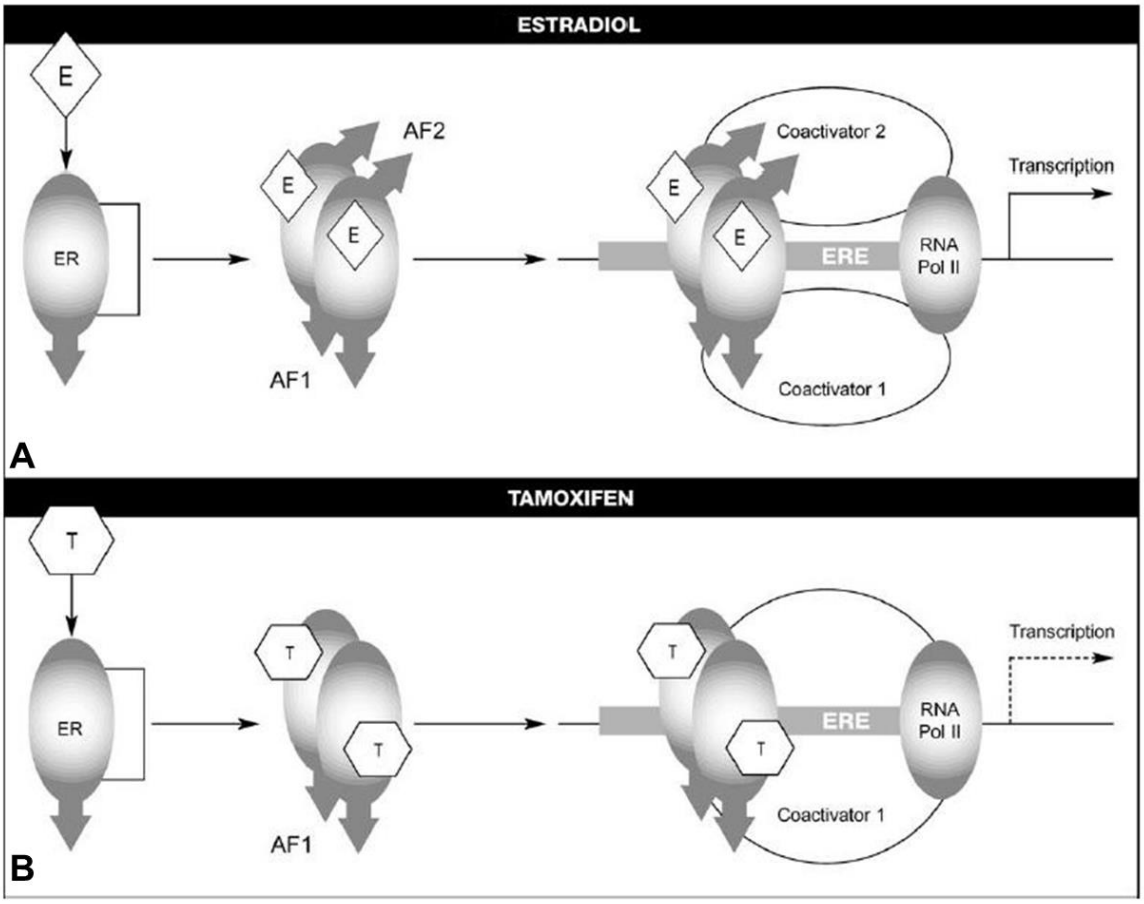

Figure 13. Mode of action of (A) E2 and (B) tamoxifen. Estrogen binds to ER, then the estrogen–ER complex homodimerizes and binds to ERE where AF1 and AF2 interact with transcriptional cofactors to activate RNA polymerase II. TAM inhibits the estrogen-ER complex formation and its binding to the ERE sensitive genes (adapted from Clemens et al, 2002 [100]).

Due to its interaction with ERs, TAM may induce some symptoms due to hypoestrogenism, such as vaginal atrophy and dyspareunia, which are common in postmenopausal women with breast cancer [101, 102]. The vaginal mucosal epithelium has both α and β ERs, with decreasing ERβ levels after menopause [103]. As shown by some authors, TAM exerts both estrogenic and anti-estrogenic effects in the vaginal epithelium of castrated rats [104]. TAM also results in a spectrum of uterine abnormalities including benign alterations such as endometrial polyps, endometrial hyperplasia, endometrial cystic atrophy, adenomyosis and uterine fibroid growth as well as malignant transformation into endometrial carcinoma and uterine sarcoma [105].

4. Endocannabinoid system and estradiol cross-talk

AEA is known to be involved in the modulation of several neuroendocrine functions, including the hypothalamic-pituitary-gonadal axis [3]. With that in mind, AEA influence in the CNS has been demonstrated through several studies, as the ability of AEA, under the control of sex hormones, to suppress the release of luteinizing hormone in OVX rats and its reversal was blocked by coadministration of AM251, a CB1 antagonist [106]. It was also observed a reduction of CB1 receptor density in the limbic forebrain, which was reversed by E2 administration in OVX rats [107]. Besides, E2 treatment after OVX reduced CB1 mRNA levels in the anterior pituitary [108]. Castrated males also exhibited a reduction in CB1 mRNA levels in the anterior pituitary, which was not reversed by administration of dihydrotestosterone, suggesting that the influence of androgens on central CB1 receptor expression is via aromatization of testosterone into E2 [108]. Endocannabinoid activity and CB1 receptor densities in the brain appear to fluctuate throughout the estrous and menstrual cycle. In the mediobasal hypothalamus of female rats, the density of CB receptors was highest during diestrus and lowest during estrus; in the limbic forebrain, the receptors affinity to cannabinoids was highest during diestrus and lowest during estrus, but their densities did not fluctuate [107]. Finally, CB1 mRNA transcript levels were found to be highest during diestrus and lowest during estrus in the anterior pituitary of rats [108].

E2 and progesterone modulate the production of AEA and its metabolizing enzymes in the rat uterus. AEA production is highest at the estrous stage and E2 and progesterone stimulates its synthesis in OVX rats [109].

After the discovery that FAAH is downregulated by sex hormones [110] it was observe that FAAH gene has an ERE [111]. It was also found a differential expression of this enzyme as well as its activity during the estrous cycle stages, in particular, the highest FAAH activity and protein content were recorded at proestrus, followed by a progressive decrease at estrus and metestrus; finally diestrus was characterized by the lowest values for both parameters [112].

5. Aims of the present study

E2 can modulate CB1 transcription as well as its expression in the CNS; FAAH expression also varies during the estrous cycle; and AEA levels and its metabolizing enzymes are affected by E2 and progesterone. These findings collectively indicate that E2 levels can influence central endocannabinoid signaling, and these can be region specific.

Uterus expresses all the ECS elements and plays a key role in the outcome of physiological events such as pregnancy. E2 is also essential for these physiological events and given its ability to induce changes in the ECS it is important to understand how this hormone regulates the ECS elements in the uterus in order to have a better understanding of the uterine physiological functions regulation. However, there are no studies showing how endocannabinoid-estrogen interacts in female reproductive tract and/or estrogen may affect the regulation of endocannabinoid system components.

With this lack of information about the endocannabinoid-estrogen interaction, the aim of the present study is to examine whether estrogen absence in OVX rats and sex steroid replacement would result in changes in the expression of the ECS elements in the uterus.

Chapter II

Material and Methods

Materials and Methods

1. Animals and treatments

Female Wistar rats from the Institute for Molecular and Cell Biology (Porto, Portugal), were maintained in standard conditions under 12 h dark/light cycle (lights on from 7 am to 7 pm) and ambient temperature of 23 °C. Food and water were available *ad libitum*. Starting at 8 weeks of age, estrous cycles were monitored daily by vaginal smear cytology. Only rats that regularly displayed consecutive 4- to 5-day estrous cycles were used. At 10 weeks of age, rats were bilaterally OVX (Fig. 14) after being anesthetized with sequential injections of promethazine (10 mg/kg body weight, s.c.) and a solution of xylazine (2.6 mg/kg body weight, i.m.) and ketamine (50 mg/kg body weight, i.m.). After 12 days of recovery, rats were allocated to one of four treatment groups that were injected s.c. with two pulses, given 24 h apart, of the following: 1) 0.1 ml oil (Oil group); 2) 10 µg EB (EB group); 3) 2 mg TAM followed, 1 h later, by 10 µg EB (TAM-EB group) or 4) 2 mg TAM (TAM group). Solutions were prepared in 0.1 ml of sesame oil. Sesame oil, EB and TAM were purchased from Sigma-Aldrich Company Ltd. (Madrid, Spain). The dose and schedule of EB administration is part of an established hormonal replacement rat model that has been used for evaluating the relation between central endocrine regulation and female sexual behavior [113-115]. Because we aimed to study the possible interference of a SERM in this model, we used TAM in a dose that is known to block partially the E2-induced progesterone-facilitated sexual behavior and the release of luteinizing hormone [116-118]. The antagonistic mode of administration of TAM, i e, 1 h before EB administration [119-121], was shown to display a good relation between the behavioral response and the modulation of ERs. All animal experimentation was conducted in agreement with accepted standards of animal care and in accordance with the European Communities Council Directives of 22 September 2010 (2010/63/EU) and Portuguese Act n 113/13.

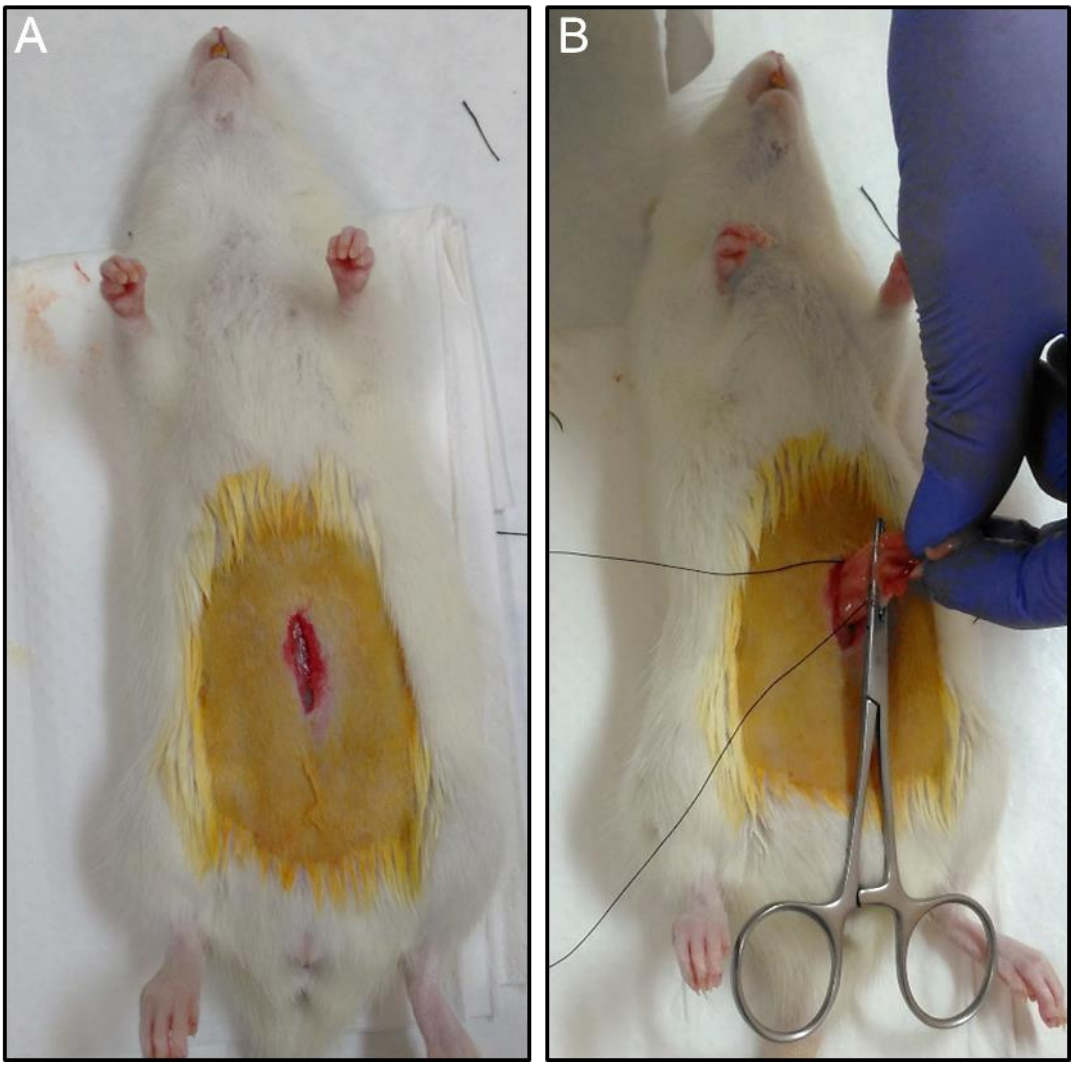

Figure 14. Ovariectomy procedure. (A) Abdominal opening followed by (B) ovaries removal and suture.

2. Tissue preparation

At the end of the experimental period, all rats from treated and control groups (n=4 per group) were ascribed for morphological studies (n = 16) and for western blot analysis (n = 16). After decapitation, the uterus was dissected, weighed and measured. Then half of each sample were quickly frozen in liquid nitrogen and stored at -80 °C for biochemical assays. The other half was immersed in a fixative solution containing 4% paraformaldehyde in phosphate buffer, pH 7.6, at 4 °C for 48h. These uterine horns were then cuted in 3 blocks and stored in a solution of 70% ethanol until they were embedded in paraffin. The 3 tissue samples of each uteri were embedded in paraffin and then transverse sections of 4 µm were cut on a microtome and mounted on poly-L-lysine coated slides. Slides were deparaffinized in xylene and rehydrated through graded ethanol solutions to water. For each animal, sets of 4 slides were stained with

hematoxylin & eosin (HE) and Masson's trichrome, and sets of 5 slides were used for immunohistochemistry. For HE staining, the slides were immersed in Meyer's hematoxylin solution (Sigma-Aldrich Company Ltd., Madrid, Spain) for 15 min, washed in tap water for 5 min, immersed in 1% eosin B (Sigma-Aldrich Company Ltd., Madrid, Spain) with 3 drops of acetic acid for 2 min, and washed in distilled water. The sections were then dehydrated with ascending ethanol passages and mounted in Entelan (Merck, Darmstadt, Germany). For Masson's trichrome staining, the slides were immersed in a solution of celestin blue (Sigma-Aldrich Company Ltd., Madrid, Spain) and left to act for 5 min. The slides were drained and, without washing, Gill II hematoxylin (Leica Biosystems, Newcastle, UK) was added for 5 min. Without washing, the slides were drained and an alcoholic picric acid solution was added to the sections for 5 min. After washing quickly in distilled water, Ponceau acid fuchsin was added for 8 min and the sections were washed again in distilled water. Then, a solution of 1% phosphomolybdic acid was added for 5 min. The sections were washed and aniline blue was added for 8 min. After washing in distilled water, the slides were air dried, dehydrated rapidly through an ascending ethanol series, and mounted in Entelan (Merck, Darmstadt, Germany).

3. Stereology

To access the variations of volume and surface area of the different layers of the uterine tissue induced by each treatment a stereological analysis was performed. The Computer Assisted Stereology Toolbox (CAST) 2.0 system from Olympus (Ballerup, Denmark) was used and all measurements were done in Masson's trichrome stained sections.

To determine the volume density of the uterus, a 32-point grid was superimposed on vertically orientated paraffin sections viewed using a 10× objective lens which enable the complete view of the section (Fig. 15). Points falling on the uteri were counted and the Cavalieri principle was applied in order to reach a volume estimate [122]:

$$V(obj) = t \times \sum a = t \times a_{(p)} \times \sum P$$

where V(obj) is the estimated uterine volume, t is the total thickness of the uterus sample (total number of sections multiplied by section thickness), a(p) is the area associated with each point, and $\sum P$ is the sum of points on sections.

Meander sampling is a function of CAST that allows random fields of view within a sample to be identified. Using the 10× objective lens, 4 fields of view on each section

were selected by meander sampling and used to determine the uteri volume density of each of the four layers (P (perimetrium), M (myometrium), E (endometrium) and L (lumen)), by point counting, using the equation:

$$V_{v(strucutre,ref)} = P_{(struct)}/P_{(total)}$$

where Vv(struct) is the volume fraction of each component, P(struct) is the number of points falling on the component and P(total) is the total number of points falling on the reference space (including the component). The component volume densities obtained were converted to absolute quantities by multiplying by total uterus volume density [122, 123].

Surface densities for the studied layers were obtained using a grid formed of cycloid arcs placed over each field of view and intercepts between the arcs and the border of the different layers were counted. The following equation was used to determine surface areas:

$$S_{(struct)} = \left(2 \times \sum \frac{I_{(struct)}}{I_{(p)}} \times \sum P_{(ref)}\right) \times V_{(ref)}$$

where I(struct) is the number of intersections of the cycloid arcs with the structure, P(ref) is the total number of points that hit the reference space, and I(p) is the length of the cycloid associated with each point in the grid [124].

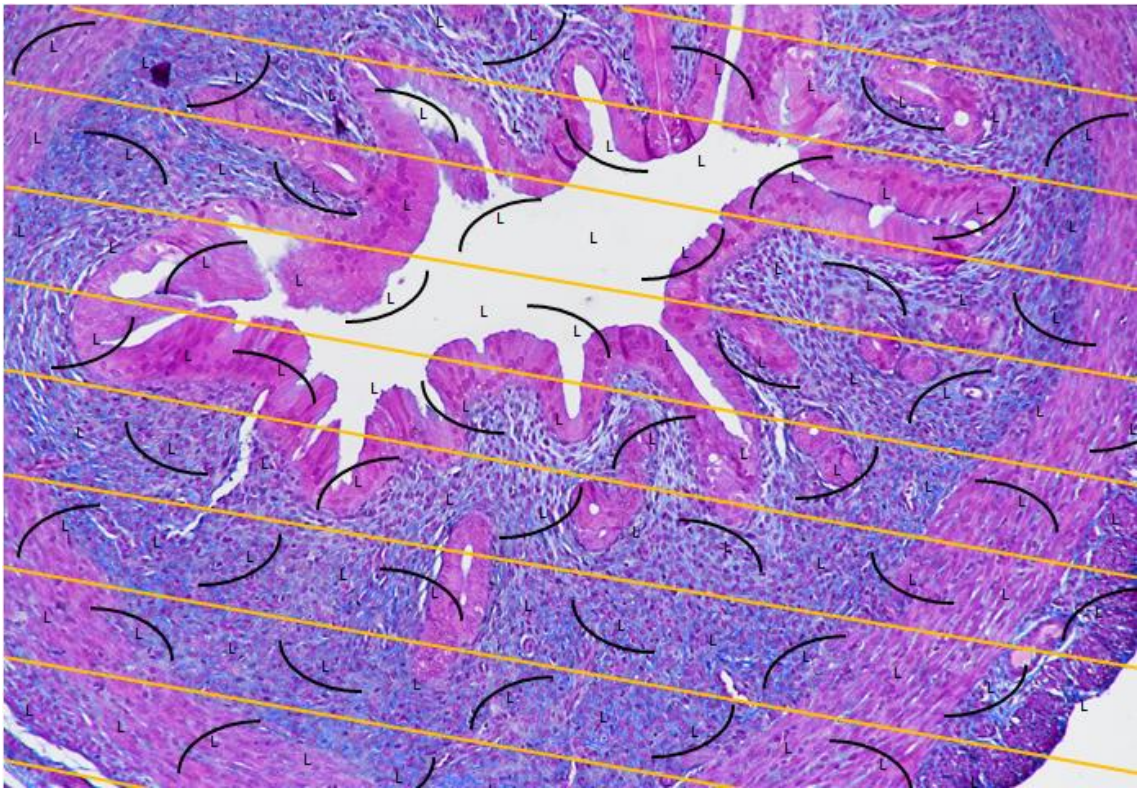

Figure 15. Example of the point and cycloid grid used for the stereological analysis.

4. Anandamide and prostaglandins extraction

4.1. Prostaglandins

Cell-free plasma (500 µl) was spiked with 20 µL of deuterated internal standard solution containing PGE_2-d4 (Cayman Chemicals) (5 mg/mL) to estimate the efficiency of the lipid extraction procedure. 0,4 g of $MgSO_4$, 0,1 g NaCl and 500 µL of ethyl acetate were added to each sample followed by a vortex homogenization and centrifugation at 3500 g for 10 min at room temperature. The upper phase was collected and dried at constant nitrogen stream. PGs where then reconstituted in 100 µL of methanol (MeOH) and transferred to an HPLC vial ready for UPLC-MS/MS analysis.

Uterine tissue samples were weighed (10-50 mg) and spiked with deuterated internal standard solution containing PGE_2-D4 (Cayman Chemicals) to estimate the efficiency of the lipid extraction procedure. 500 µL of MeOH where added to each eppendorf containing the uterine tissue as well as C18 powder (1-2 g) to improve lipid extraction. Samples where homogenized using pellet pestle and centrifuged at 800 g for 30 min at room temperature. The upper phase was collected dried at constant nitrogen stream and then reconstituted in 80 µL of acetonitrile (ACN) and transferred to an HPLC vial ready for UPLC-MS/MS analysis.

4.2. Anandamide

For the extraction of AEA from cell-free plasma was applied the same protocol applied for PGs.

Uterine tissue was weighed (10-50 mg) and spiked with 20 µL of deuterated internal standard solution containing AEA-d4 (Cayman Chemicals) (0,001 mg/mL) to estimate the efficiency of the lipid extraction procedure. 300 µL of MeOH/ACN (1:1) where added to the eppendorfs containing the sample as well as the internal standard. The mix was then homogenized using pellet pestle followed by 10 min of sonication. After that, the eppendorfs were centrifuged at 800g for 90 sec at room temperature and the upper phase was collected to a vial. One more cycle of extraction solution, homogenization, centrifugation and upper phase collection was performed. The upper phase was then dried at constant nitrogen stream and then reconstituted in 100 µL of MeOH followed by its transfer to an HPLC vial ready for UPLC-MS/MS analysis.

5. Quantification of prostaglandins and anandamide

Separation and quantification of the target analytes were performed by using a liquid chromatography Acquit UPLC system interfaced to a triple quadrupole mass selective detector Micromass Quattro micro API™ (Waters, Milford, MA, USA). The injection volume was set at 10 µL.

For PGs quantification, the mobile phases were (A) 5% ammonium acetate (5 mM) in ultra-pure MilliQ water and (B) MeOH (UPLC grade, VWR, Radnor, PA) in an isocratic gradient. Mass spectrometry analysis was performed with an electrospray ionization (ESI) source in the negative (ESI-) ion mode for all the analytes. Nitrogen was used as the nebulizer gas. The optimum mass spectrometry parameters were: capillary, 3.00 kV; extractor, 2 V; RF Lens, 0.5 V; Source Temperature, 150ºC; Desolvation Temperature, 350ºC; Desolvation Gas Flow, 350.0 L/h; Cone Gas Flow, 60.0 L/h; LM Resolution, 13.0; Ion energy, 1.0; Entrance, 1; Exit, 2; Multiplier, 650. All analyses were done in multiple reaction monitoring mode. The UPLC-MS/MS settings and ESI source parameters were optimized by manual infusion of standards with a syringe pump and acquisition of the respective daughter spectrum after collision in the quadrupole (Fig. 16).

For AEA quantification, the two mobile phases consisted of 2 mM ammonium acetate, in ultra-pure MilliQ water, containing 0,1% formic acid (A) and acetonitrile (UPLC grade, VWR, Radnor, PA) containing 0,1% formic acid (B). Gradient conditions were as follows: 0-0.5 min, 85% A; 0.5-3.5 min, 85-30% A; 3.5-8.0 min, 30-1% A; 8.0-11.0 min, 1% A; and then reequilibrated at 85% A until 15.0 min. Quantification of analytes was achieved using tandem electrospray mass spectrometry in positive ion mode (ESI+) with the products being monitored in multiple reaction monitoring mode. The transitions used for AEA and AEA-d4 were m/z 348 > 62 (Fig. 16) and m/z 352 > 66.

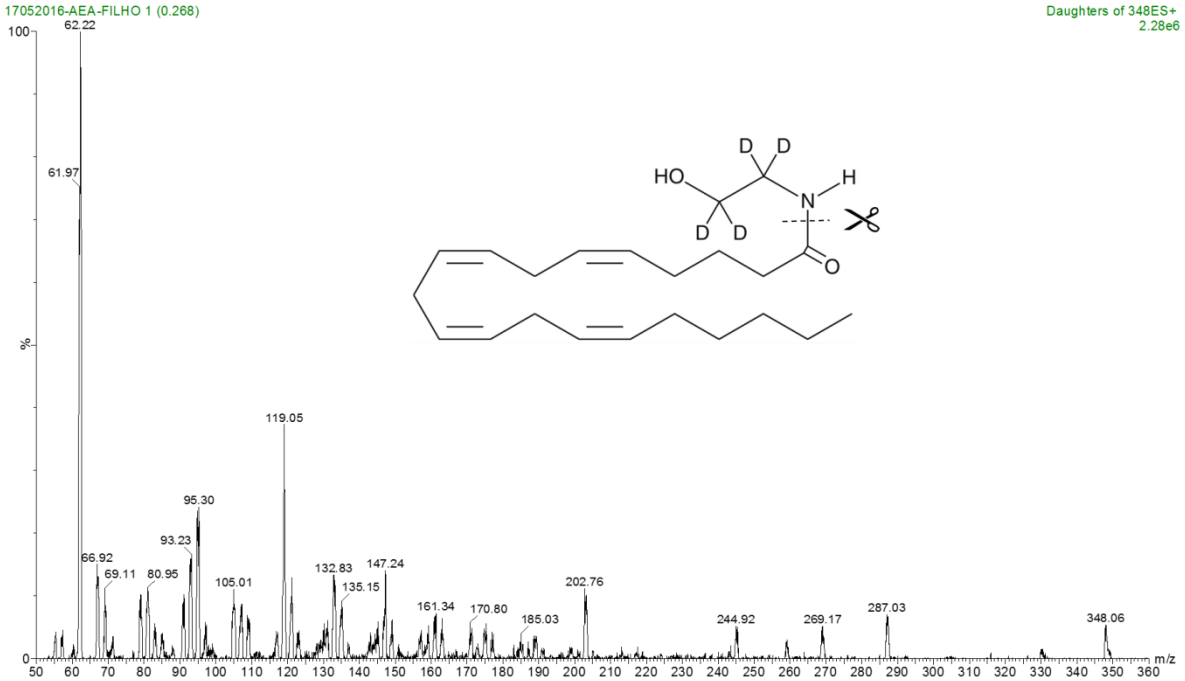

Figure 16. Daughter ion scan. Conditions were collision 20 and cone 30.

6. qRT-PCR analysis

In order to identify changes in the transcriptional levels of CB1, CB2, FAAH, NAPE-PLD and COX-2 in the different treatments, it was performed a semi quantitative real-time polymerase chain reaction (qRT-PCR) technique.

Uterine tissue was subject to homogenization with 500 µL of TRIsure reagent (Bioline, London, UK). After 5 min of incubation at room temperature, 100 µL of chloroform were added to each sample to separate the sample in three phases: an upper aquous one, which contains RNA, an interphase which contains DNA and an organic phase which contains proteins. After RNA isolation, it was performed a precipitation with 250 µL isopropanol 100%. The pellet was then submitted to air dry followed by a wash with ethanol 70%. RNA samples were ressupended and the RNA was quantified

spectrophometrically at 260 and 280 nm using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) followed by conversion to cDNA using the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, USA). cDNA was amplified with specific primers, using KAPA SYBR® FAST qPCR Master Mix 2 Kit (Kapa Biosystems, Woburn, MA, USA) in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, USA), according to the kit protocol. Each reaction was run in duplicate.

For Q-PCR, 10 pmol of gene-specific primers (Table. 1) was used in a SYBR green system (Roche Diagnostics, Lewes, UK) with 1 μl of cDNA as template in a Roche Lightcycler 1.2. The PCR conditions were, in all cases, initiated with a denaturation step at 95 C for 10 min, followed by up to 50 cycles of denaturation, annealing and primer extension as described in Table 1 and the expression levels corrected for the levels of rat β-actin using the 2-ΔΔCt method [125] with the data normalized to Oil group.

Table 1. Primer sequences, gene accession number, and Q-PCR conditions.

mRNA target	Primers (5'-3')	Conditions	Reference
Rat B-Actin	S-CCTAGCACCATGAAGATCAA AS-TTTCTGCGCAAGTTAGGTTTT	95 °C, 15 sec 60° C, 20 sec 72 °C, 20 sec	[126]
CB1	S-CATCATCATCCACACGTCAG AS-ATGCTGTTGTCTAGAGGCTG	95 °C, 15 sec 60 °C, 30 sec 72 °C, 20 sec	[127]
CB2	S-TTTCCCACTGATCCCTAACG AS-AGTTAACAAGGCACAGCATG	95 °C, 15 sec 60 °C, 30 sec 72 °C, 20 sec	[127]
NAPE-PLD	S-GTCACAACCACTACGACCAC AS-AAGCAGGGCAGTAACCAG	95 °C, 15 sec 58 °C, 30 sec 72 °C, 20 sec	[126]
FAAH	S-CCTAGCACCATGAAGATCAA AS-TTTCTGCGCAAGTTAGGTTTT	95 °C, 15 sec 60 °C, 30 sec 72 °C, 20 sec	[126]
Cox-2	S-CTGAGGGGTTACCACTTCCA AS-TGAGCAAGTCCGTGTTCAAG	95 °C, 15 sec 58 °C, 20 sec 72 °C, 20 sec	[126]

7. Western blot analysis

To detect changes in CB1, CB2, FAAH, NAPE-PLD and COX-2 expression levels after the respective treatments, it was performed a western blot technique.

Uterine tissue (100 mg) was dissected in 300 µL of homogenization buffer (20 mM HEPES, 2 mM EDTA, 10 mM KCl, 1,5 mM $MgCl_2$ and protease inhibitor cocktail 1%) and the dissected tissue was homogenized in a potter. After centrifugation of the protein extracts at 700 g for 10 min, the supernatant was collected and the protein concentration of each sample was determined by the Bradford method.

Protein samples (25 µg) were prepared in sample buffer, denatured by boiling for 3 min, subjected to SDS–polyacrylamide (10%) and transferred onto nitrocellulose membranes for 50 minutes in a semi dry transfer system (Trans-Blot SD semi dry transfer cell, Bio-Rad). After blocking non-specific binding sites with blocking buffer (5% of dry milk in phosphate buffer (PBS) with Triton X-100, 0.1%), the membranes were incubated with the primary antibodies anti-CB1 (1:500 sc-10066, St.Cruz Biotechnology, CA, USA), anti-CB2 (1:200 sc-25494, St.Cruz Biotechnology, CA, USA), anti-FAAH (1:200 sc-26427, St.Cruz Biotechnology, CA, USA), anti-NAPE-PLD (1:200 sc-163117, St.Cruz Biotechnology, CA, USA) and anti-COX-2 (1:200 sc-23984, St.Cruz Biotechnology, CA,

USA) overnight at 4 °C. Then, after washed with PBS 0.1% Triton, the membranes were incubated for 1 h, at room temperature, with peroxidase-conjugated secondary antibody (1:5000). Membranes were exposed to chemiluminescent substrate Super Signal West Pico and immunoreactive bands were visualized by ChemiDocTM Touch Imaging System (BioRad, Laboratories Melville, NY, USA). The membranes were then stripped and incubated with anti-β-tubulin (1:500 sc-9104, Santa Cruz, CA, USA) for a loading control.

8. Immunohistochemistry

Expression of CB1, CB2, NAPE-PLD, FAAH and COX-2 was analyzed using an avidin-biotin peroxidase complex immunohistochemical technique (Vector laboratories, Peterborough, UK). After dewaxing and rehydration, antigen recovery was performed with citrate buffer and microwave followed by 30 min of incubation with sodium borohydride to remove unbound aldehydes. The blocking of non-specific binding sites was performed with serum from the species where the secondary antibody was generated. Slides were then incubated overnight with the primary antibodies rabbit anti-CB2 (1:200) and goat anti-CB1 (1:200), FAAH (1:100), NAPE-PLD (1:100) and COX-2 (1:100) at 4 °C. After washing with PBS they were incubated with diluted biotinylated secondary antibody for 1h, followed by incubation with ABC-peroxidase reagent after another PBS wash. The reaction was developed by incubation with 3,3'-diaminobenzidin. Negative controls were performed with the inclusion of blocking serum instead of primary antibodies. The slides were counterstained with Mayers hematoxylin solution (Sigma-Aldrich company Ltd, Madrid, Spain) and mounted in Entelan mounting medium (Merck, Darmstadt, Germany), and then used for the qualitative analyses of immunohistochemistry staining and cellular distribution of each enzyme and receptor.

9. Statistical analysis

Statistical analysis was carried out by ANOVA, followed by the Tukey post hoc test to make pairwise comparisons of individual means when significance was indicated (GraphPad PRISM v. 6.0, GraphPad Software, Inc., San Diego, CA, USA). The results are the mean of at least three independent experiments carried out in triplicate. Data are expressed as the mean±SEM, and differences were considered to be statistically significant at $p < 0.05$.

Chapter III

Results

Results

1. Estradiol and Tamoxifen effect in uterus morphology

1.1. Overall morphology

Figure 17. Overall uterine morphology in different treatments after formaldehyde preservation. As seen in the comparisons, EB causes uterus hypertrophy contrary to TAM treatment. TAM+EB treatment also induces hypertrophy but not as significant as EB treatment.

To investigate the impact of different treatments in the uterus, it was first observed the overall morphology of the uterus (Fig.17). EB treatment led to an increase in the uterus size but no change was observed in rats treated with TAM. TAM+EB administration also produced an increase in uterine size that however, did not reach EB levels. These observations are supported by the uterine weight determined upon the different treatments (Fig.18). Just like observed macroscopically, EB induced an increase in uterus weight of almost 3× when compared to Oil and TAM treatment and 2× higher than TAM+EB treatment. This data shows that the EB-dependent increase in uterine weight is inhibited by prior TAM administration.

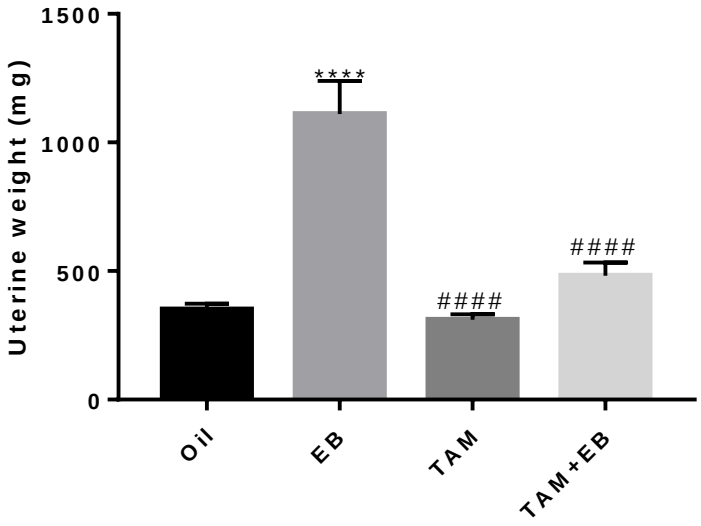

Figure 18. Uterine weight of the rats submitted to different treatments. Significant differences between Oil and other treatments are denoted by **** ($p < 0.0001$) and between EB and other treatments are denoted by #### ($p < 0.0001$).

1.2. Histology of the uterus

Contrary to the human, the rat uterus is composed by two uterine horns, as is seen in the overall morphology (Fig. 17). It has an outer serosa layer named perimetrium, and two muscle layers called myometrium, being the outer longitudinal and the inner transversal muscle fibers. Close to the muscle layer there is the endometrium, which contains the glands and is the layer adjacent to the lumen which is constituted by epithelial columnar cells (Fig. 19).

To evaluate morphological alterations at the tissue level triggered by EB and TAM, the tissue sections were stained with HE, which allowed studying cell morphology. With this stain it was observed that EB treatment produced more developed luminal and glandular epithelium, but no other alterations were evidenced at the different layers that constitute the uterus. It was also performed a Masson's trichrome technique, which permits a better differentiation between muscle and collagen fibers and therefore, also a better layer differentiation (Fig. 20).

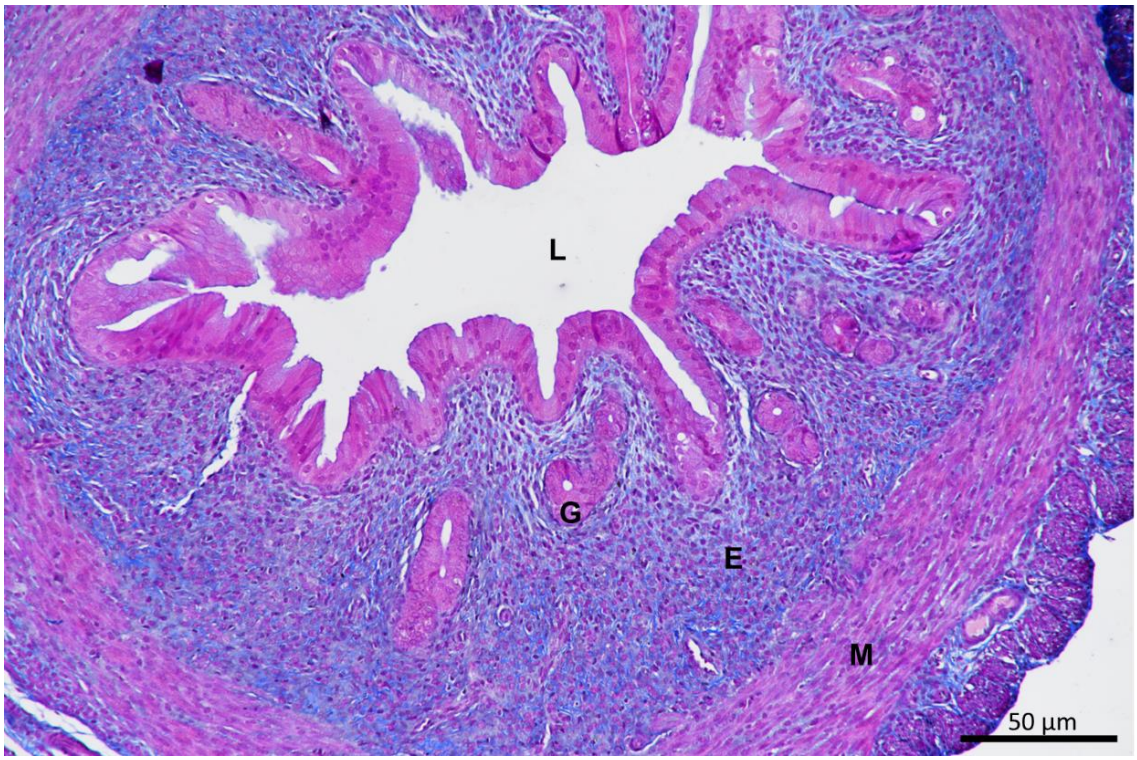

Figure 19. Masson's trichrome staining of a rat uterus. It has an outer serosa layer named perimetrium, and two muscle layers called myometrium (M), followed by endometrium (E), which contains the glands (G) and is adjacent to the lumen (L).

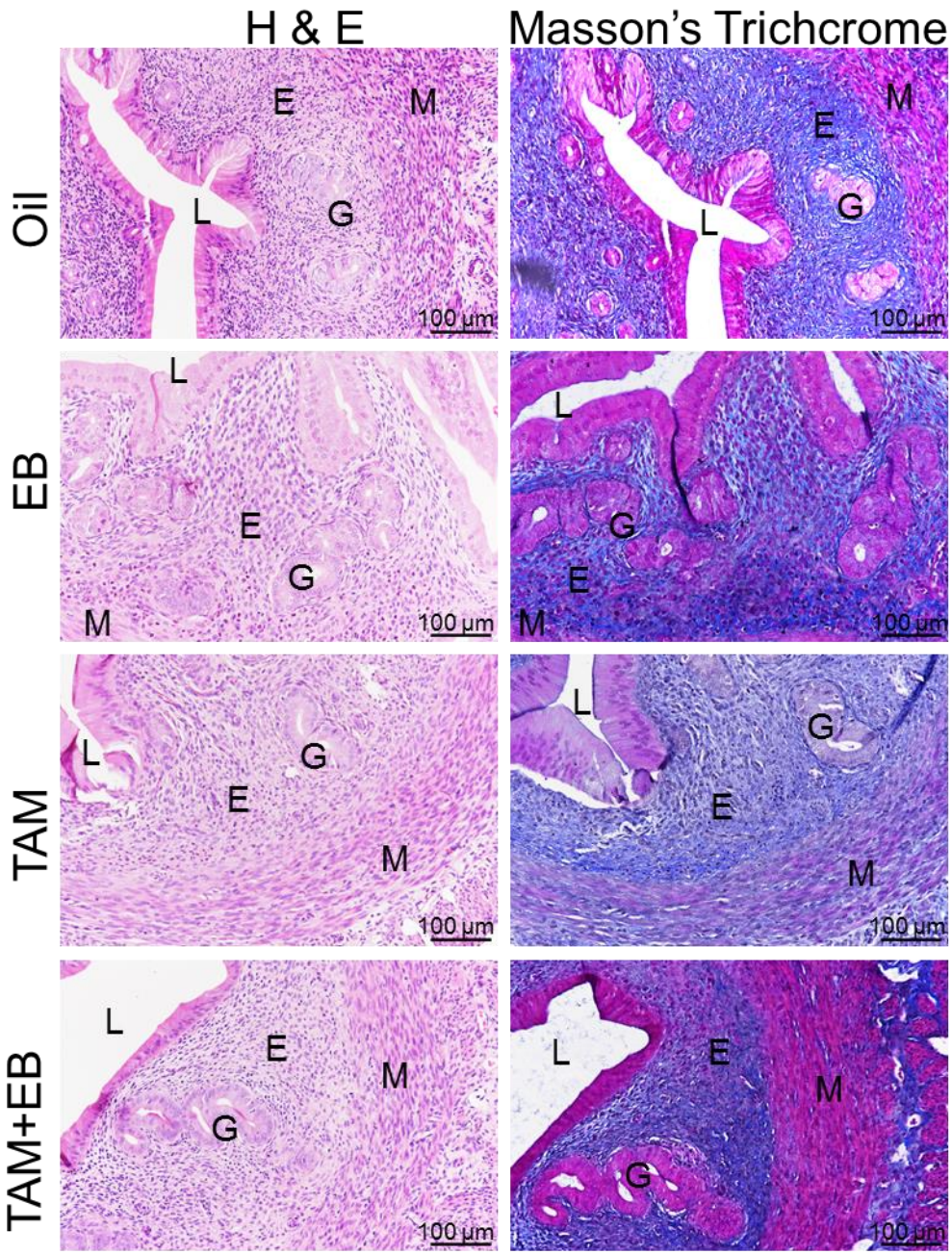

Figure 20. Hematoxylin & eosin and Masson's trichrome staining in the four different treatments. Myometrium (M), Endometrium (E), Gland (G), Lumen (L).

1.3. Stereology

In order to study the effect of the treatments in the different layers that constitute the uterus as well as the effect in the glands, a stereology analysis was performed. For that, the points and cycloid interceptions with the perimetrium, myometrium, endometrium and lumen where counted to obtain the relative volume (Fig. 21) and surface density (Fig. 22) of each layer. The same analysis was made for glands (Fig. 23 and 24).

The relative volume estimations showed that upon EB treatment there is a decrease in endometrium volume which is compensated by an increase of myometrium. In this study it was determined the relative volume of uterine layers, however, changes in uterine weight, that are correlated, allow us to conclude that all the layers in the EB treatment had increased its volume but the myometrium was the layer that had a proportionally higher increase and the endometrium the one that had a proportionally lesser increase. The TAM and TAM+EB treated rats displayed no changes in relative volume compared to Oil group which shows that TAM alone has no estrogenic abilities in the uterus structure and that EB administration was not able to overcome the TAM-induced atrophy.

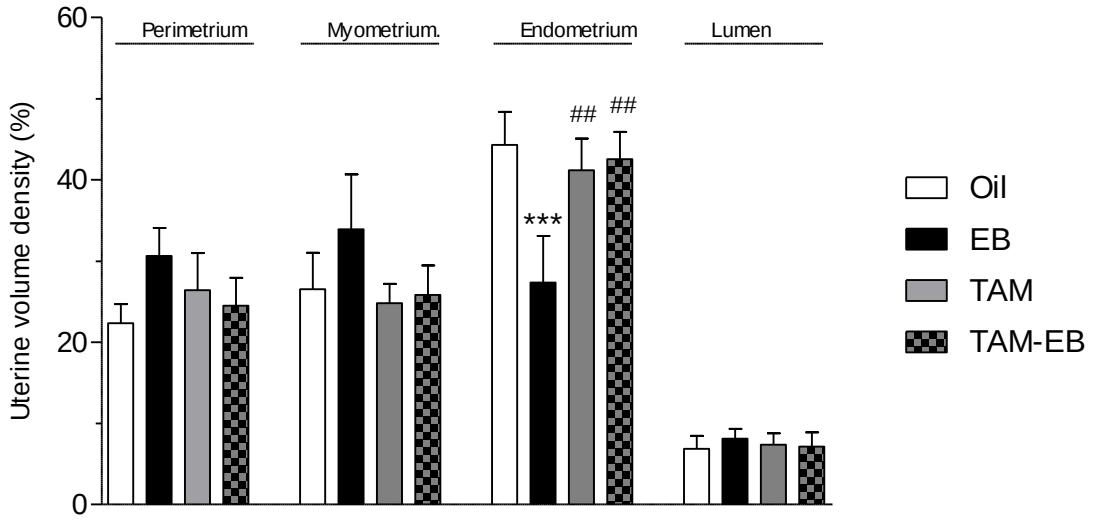

Figure 21. Uterine volume density of each of its layers in the different treatments. Significant differences between Oil and other treatments in the endometrium are denoted by *** ($p < 0.001$) and between EB and other treatments are denoted by ## ($p < 0.01$).

Oil and TAM treatment induced a significant increase in the surface density of myometrium compared to EB. TAM+EB rats also showed a higher, but not significant, myometrium surface density than EB treated rats. This result allows to conclude that the myometrium of EB-treated rats increased due to an overall expansion of the tissue because its volume has increased and its surface area has decreased.

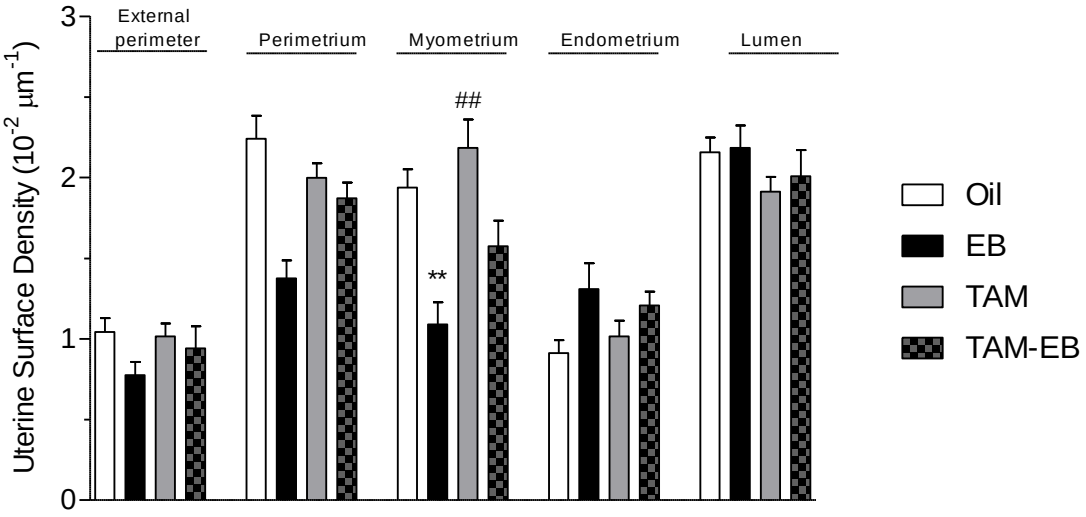

Figure 22. Surface density of each uterine layer in the different treatments. Significant differences between Oil and other treatments in the myometrium are denoted by ** ($p < 0.01$) and between EB and other treatments are denoted by ## ($p < 0.01$).

Glandular volume analysis revealed that EB, TAM and TAM+EB treatments induced an increase in glandular volume compared to Oil administration (Fig. 23). The surface density of the glands displayed a decrease upon EB and TAM+EB treatments compared to Oil treatment but no significant decrease was observed with TAM treatment. This observations, together with the volume and surface area analysis suggest that with in EB and TAM+EB treated rats, the glands grow, which can be due to some swelling or hyperplasia. However, no differences were found regarding the number of glands per volume of endometrium (Fig. 24).

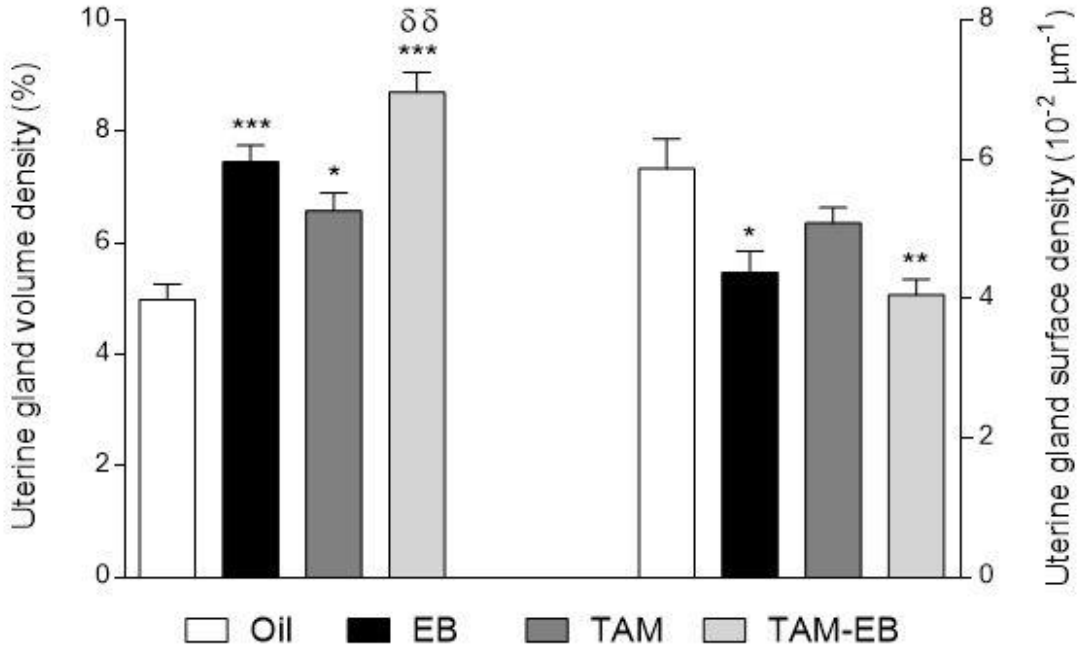

Figure 23. Volume and surface density of uterine glands in the different treatments. Significant differences between Oil and other treatments in the myometrium are denoted by * ($p < 0.05$) or *** ($p < 0.001$) and between TAM and other treatments are denoted by δδ ($p < 0.01$).

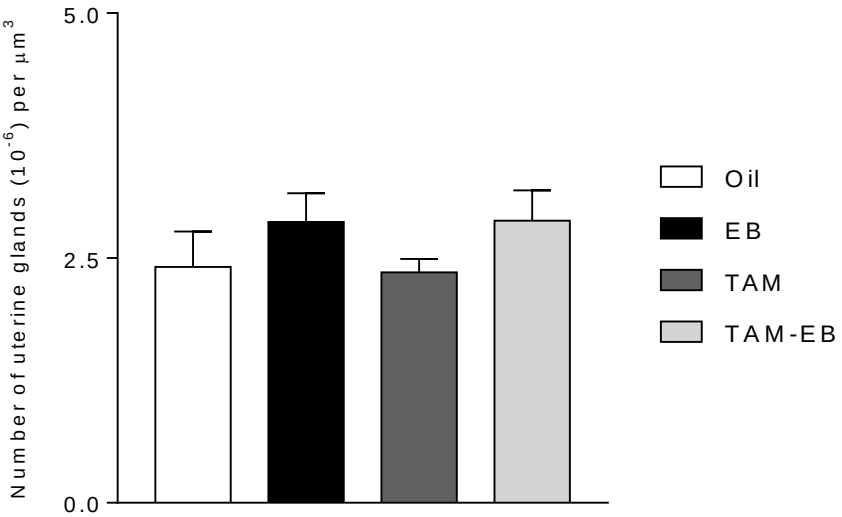

Figure 24. Uterine density glands per volume of endometrium in the different treatments.

2. Estradiol effect on cannabinoid receptors and AEA-metabolic enzymes

2.1. Immunohistochemistry

To observe the cells that expressed the receptors of the endocannabinoid system and the AEA-metabolic enzymes and see if there are changes in its expression in the different treatments, immunohistochemistry was performed (Fig. 25 and 26).

In figure 25, it can be seen that the CB1 presents mainly epithelial expression in both luminal and glandular cells, but is also present in the muscle cells of myometrium. Similar expression was observed for CB2. However, no differences in staining where detected in both cannabinoid receptors between treatments.

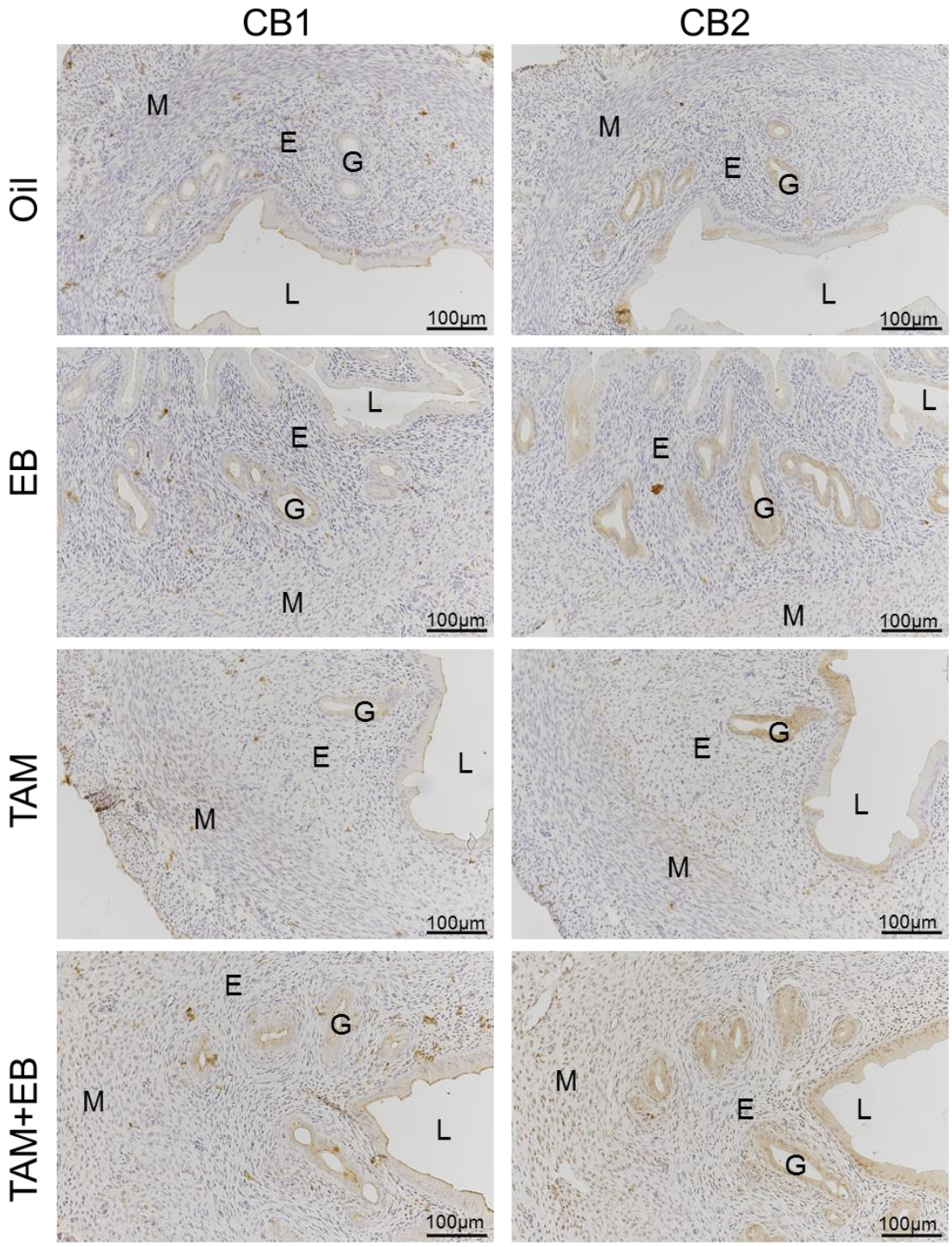

Figure 25. Immunohistochemistry staining of CB1 and CB2 in the four different treatments. Myometrium (M), Endometrium (E), Gland (G), Lumen (L).

As the receptors, AEA-metabolic enzymes showed the same sites of expression, that is, in the luminal and glandular cells and muscle cells of myometrium (Fig. 26). Yet no changes were observed in the different treatments. COX-2 was also revealed to be

expressed in the epithelial and muscle cells. No differential expression was detected in the different treatments.

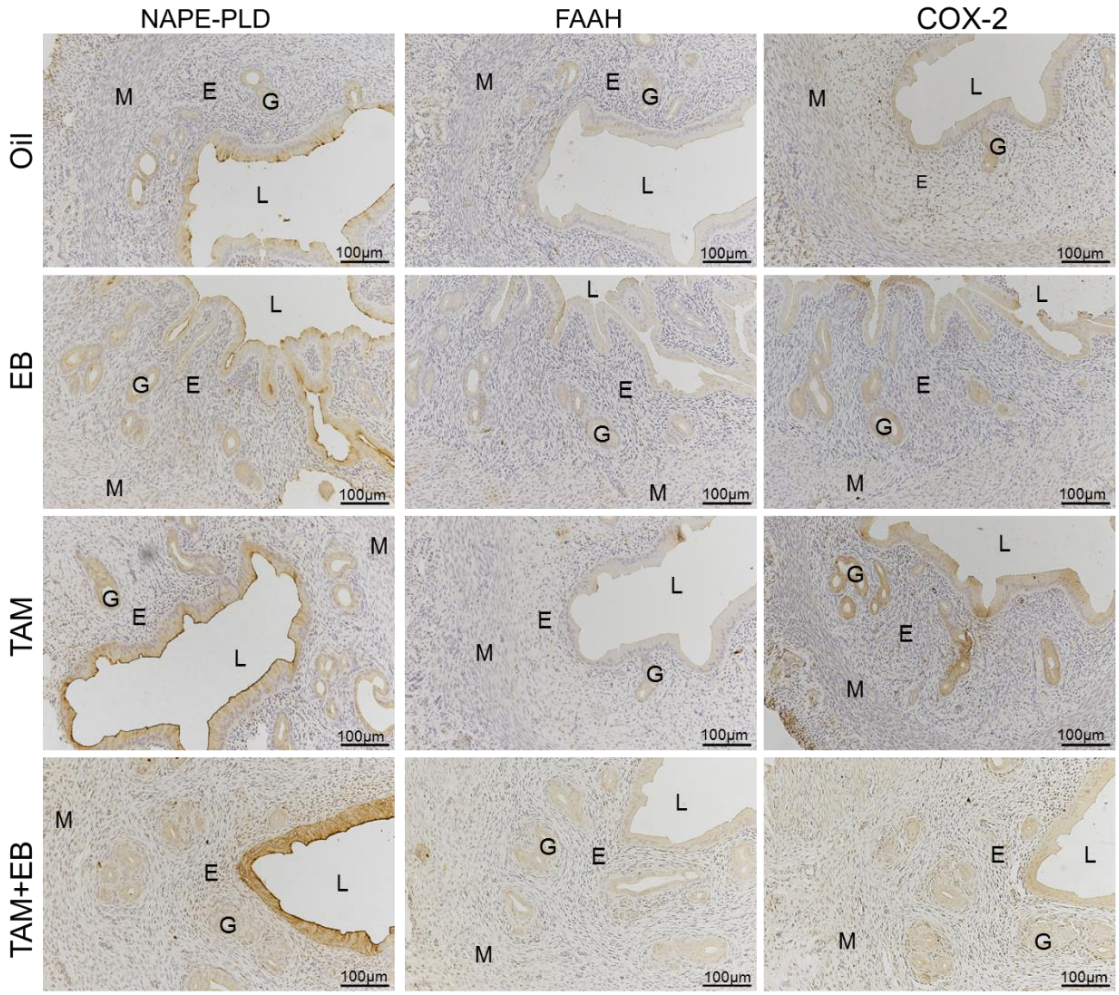

Figure 26. Immunohistochemistry staining of NAPE-PLD, FAAH and COX-2 in the four different treatments. Myometrium (M), Endometrium (E), Gland (G), Lumen (L).

2.2. Western blot analysis and qRT-PCR

To examine the capacity of EB, TAM and TAM+EB treatments do induce changes in the transcription as well as in the expression of the cannabinoid receptors and AEA-metabolic enzymes, real-time PCR and Western blot analysis was performed. Densitometric analysis of the bands in the western blots was evaluated. In figure 27 the densitometric analysis shows an increase in CB1 and CB2 caused by EB administration. Contrary to EB, TAM treatment showed similar levels of expression compared to the Oil group. It was also observed that EB could not reverse the effects caused by TAM in both

CB1 and CB2. Altogether this data confirms that the expression of the cannabinoid receptors is estrogen receptor dependent.

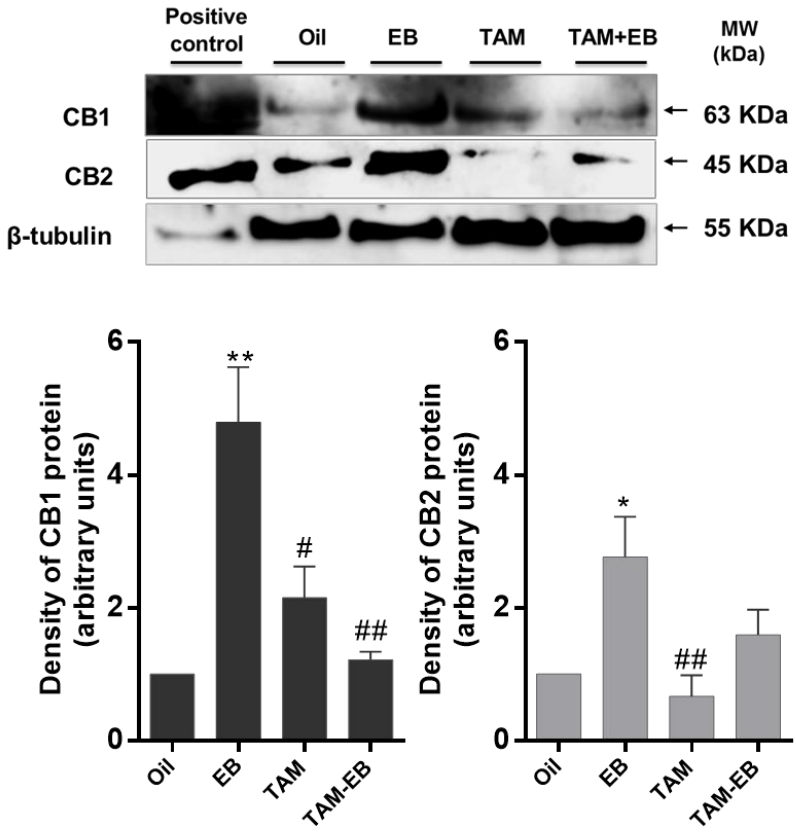

Figure 27. Western blot and respective densitometries of CB1 and CB2. β-tubulin was used for loading control in western blot. Significant differences between the oil and other treatments are denoted by * ($p < 0.05$) or ** ($p < 0.01$) and between EB and TAM and TAM+EB are denoted by # ($p < 0.05$) or ## ($p < 0.01$).

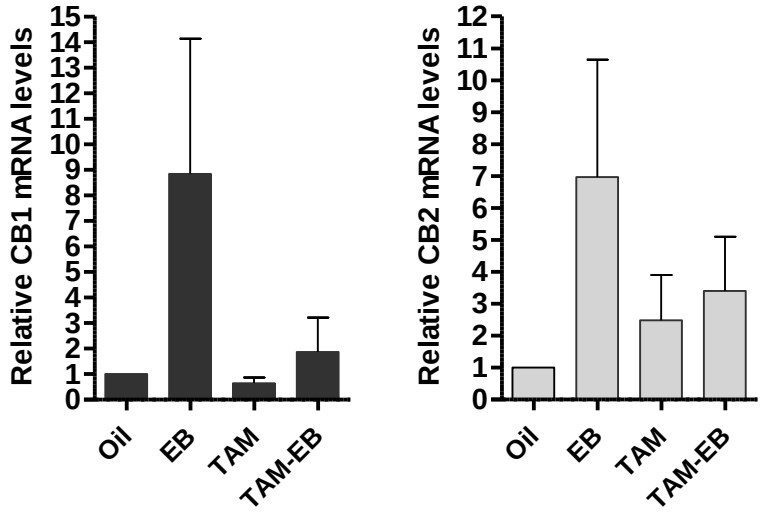

Figure 28. qRT-PCR of CB1 and CB2. β-Actin was used as housekeeping.

RT-PCR analysis of the cannabinoid receptors (Fig. 28) support the western blot results, which display higher levels of the receptors transcription with the administration of EB even though they are not significant. Similar levels in the TAM and TAM+EB treatments were obtained when compared to the Oil group.

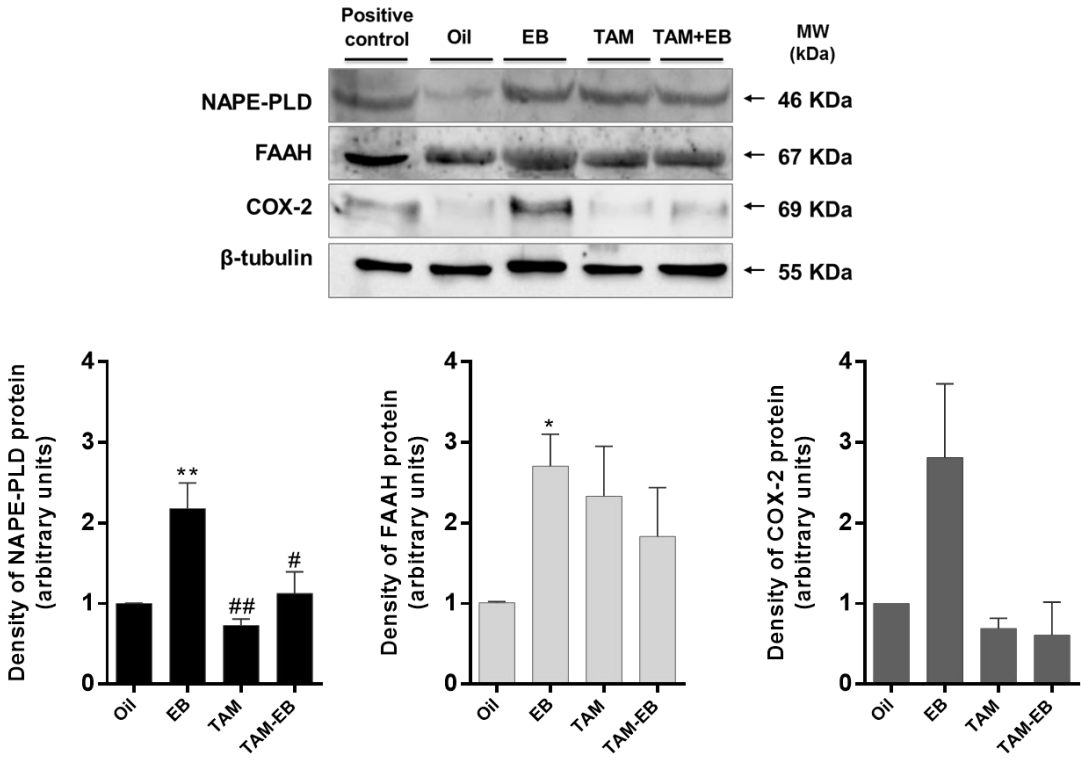

Figure 29. Western blot and respective densitometries of NAPE-PLD, FAAH and COX-2 (preliminary data). β-tubulin was used for loading control. Significant differences between the oil and other treatments are denoted by * ($p < 0.05$) or ** ($p < 0.01$) and between EB and TAM and TAM+EB groups are denoted by # ($p < 0.05$) or ## ($p < 0.01$).

As displayed in figure 29, in the same line as the receptors, NAPE-PLD and FAAH are expressed in higher levels when EB is administrated. Similarly, to CB1 and CB2, NAPE-PLD showed similar levels of expression upon TAM treatment in comparison to the Oil group. Moreover, EB could not revert the effect caused by TAM. A diverse pattern was induced by TAM in FAAH which showed an increase in the expression levels compared to the Oil group that could not be reverted by a posterior EB administration.

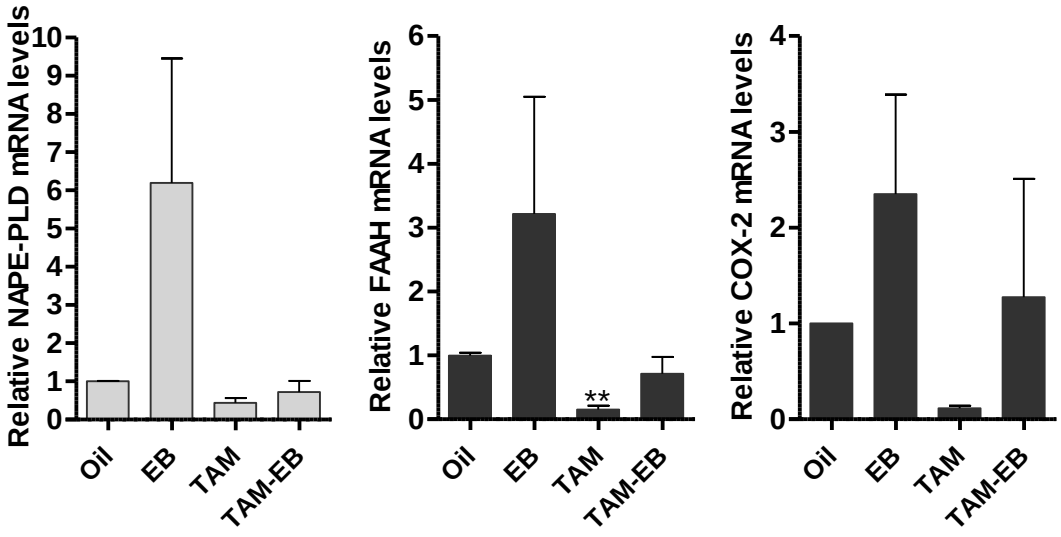

Figure 30. qRT-PCR of NAPE-PLD, FAAH and COX-2 (preliminary data). β-Actin was used for RT-PCR housekeeping. Significant differences between the Oil and treated uteri are denoted by ** ($p < 0.01$).

The RT-PCR of NAPE-PLD exhibited a comparable pattern to the obtained by western blot. However, FAAH transcript levels in the TAM and TAM+EB groups were not coincident with protein levels. The results were similar to the Oil transcriptional levels. These results illustrate that, like the cannabinoid receptors, the AEA-metabolic enzymes transcription and expression occurs in an ER-dependent way. Transcription of COX-2 gene was also increased with EB treatment and decreased with TAM showing that also COX-2 seems to be influenced in an estrogen-dependent manner.

2.3. UPLC-MS/MS quantification

2.3.1.Prostaglandins

To investigate the effects of three different treatments in PGE_2 and $PGF_{2\alpha}$ levels in plasma and uterus, the samples where submitted to a lipid extraction followed by UPLC-MS/MS quantification. It wasn't observed any statistical differences in both PGs in plasma with different treatments but, as shown in figure 31, the plasmatic levels of PGE_2 were higher than the $PGF_{2\alpha}$ concentration, independently of the treatment. Uterine PG levels, showed a decrease in PGE_2 due to EB treatment but no differences were found in the $PGF_{2\alpha}$ levels. Moreover, no correlation was found between the plasmatic and uterine levels of both PGs. However, as shown in figure 31 the levels of PGE_2 were, independently of the treatment, always 10× higher than $PGF_{2\alpha}$ in the plasma. Contrary, in the uterus the levels of $PGF_{2\alpha}$ were around 100× higher than PGE_2.

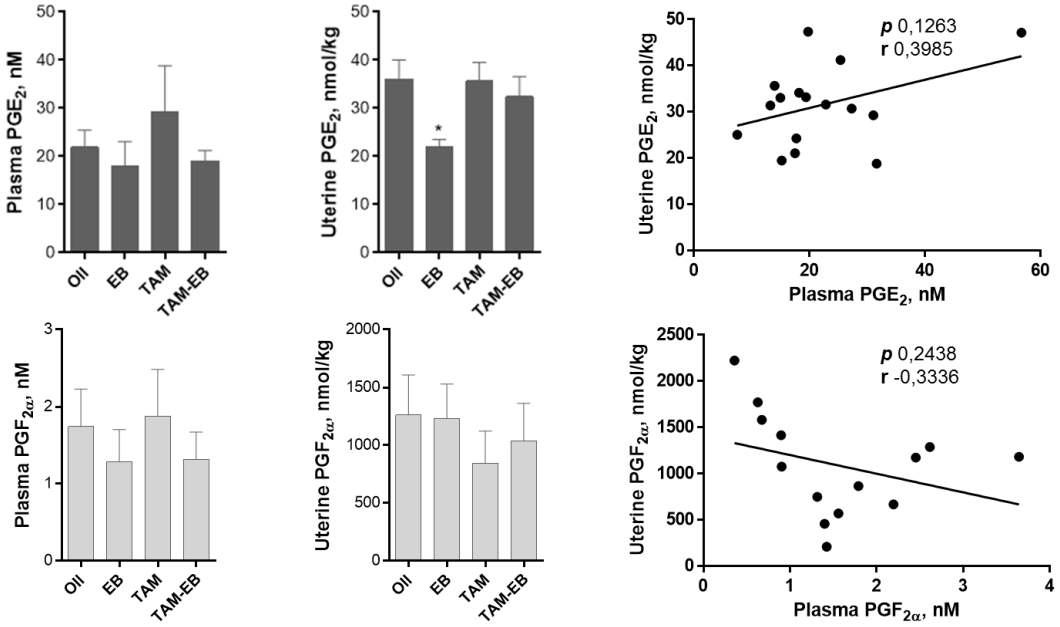

Figure 31. Effect of EB, TAM and TAM+EB in PGE_2 and $PGF_{2\alpha}$ levels in plasma and uterus. Significant differences between oil and the different treatments are denoted as * ($p < 0.05$).

2.3.2. Anandamide

Plasmatic and uterine AEA was quantified also by UPLC-MS/MS. The results obtained showed that EB treatment induced an increase of plasmatic AEA (Fig. 32). Uterine AEA quantification revealed no differences independently of the treatment. Like PGs, no correlation was found between uterine and plasmatic levels of AEA.

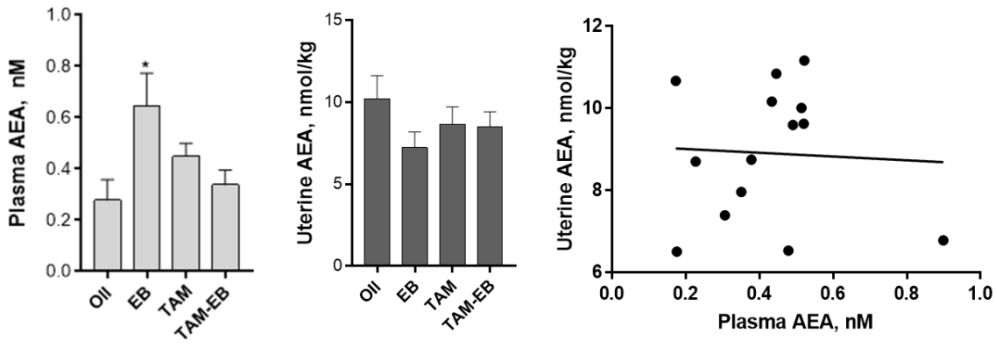

Figure 32. Effect of EB, TAM and TAM+EB in AEA levels in plasma and uterus. Significant differences between Oil and the different treatments are denoted as * ($p < 0.05$).

Chapter IV

Discussion

Discussion

The ECS is involved in several physiological events including modulation of implantation and pregnancy establishment. The regulation of these functions by eCBs has resulted in a rising interest in ECS in part due to its pharmacological potential. Moreover, because the uterine AEA levels must be tightly regulated to create proper embryo implantation conditions, thus AEA-metabolic enzymes expression must be highly controlled.

Because of the great cannabinoid receptor and enzyme expression distribution in the CNS and the tight regulation of endocannabinoid system, the hypothalamic-pituitary-gonadal axis are of great importance in this system, since it represents the central regulation of the peripheral gonadal function and its influence in uterine morphology and physiology. In fact, it has been showed that E2 has the ability to influence the expression of the ECS elements in the CNS and that ECS elements also suffer variations during the rat hormonal cycle.

In order to study the involvement of E2 in the regulation of ECS we have employed a model of E2 deprivation, which consisted of ovariectomy followed by exogenous administration of the hormone and/ or of a known modulator of ER action. In this study, these animals were submitted to four different treatments being Oil, EB, TAM and TAM followed by EB. Previous studies have shown the ability of E2 to stimulate epithelial growth in the uterus as well as general edema [128]. Here, we observed that EB had the ability to produce an increase of uterine weight and changes in its overall morphology, which is in accordance with previous studies about EB effects in uterine morphology [129]. Furthermore, present results show that, when administered alone TAM had no effect in uterine weight and morphology, which may be due to its inability to activate ERα, the receptor known to be involved in E2-dependent uterotropic changes. Moreover, when TAM is administrated in its antagonistic mode, preceding EB administration, it was able to prevent EB-induced changes in uterine morphology

Histological analysis by HE and Mason's trichrome presented no visible differences in uterine structure upon the four treatments, although it is suggestive of an increase in gland diversity. Contrariwise, the stereology analysis showed that EB induced an increase in relative uterine volume that was accompanied with decreased surface density, showing that EB induced uterine growth by increasing cellular proliferation and hyperplasia, which is further corroborated by increased uterine weight upon EB treatment. It was also observed that EB induced an increase in the glandular volume and with decreased surface density, which is in accordance with previous studies showing

that uterine epithelial cells proliferation is controlled by estrogens through activation of ERα [130], and that it is essential for pregnancy events, like implantation and decidualization [131]. Present results also suggest that the previously reposted E2-induced increase in glandular secretion is accompanied by increased glandular proliferation. Interestingly, TAM administrated alone was also able to increase glandular volume but did not induced a decrease in surface area suggesting that TAM induces glands to expand but does not induce proliferation. This result can be explained by the partial agonist effect of TAM in the uterus [128], and may help explain the reported differences seen in patients submitted to TAM treatment that sometimes show glandular hyperplasia ranging from simple to complex [132]. When administrated prior to EB, TAM did not prevent the EB-induced growth and cell proliferation in uterine glands, which may be due to ER activation by both EB and TAM. Nevertheless, the higher increase in volume density without an accompanied higher decrease in surface area suggests that TAM was able to partially reduce EB-induced cell proliferation, which may be suggestive of less functional glands.

Since E2 is essential for uterine physiological functions and influence ECS elements in the CNS as well as in other organs, the direct effect of E2 in the expression and transcriptional levels of these elements was studied. It was previously described by our lab that ECS elements could be found in differentiated endometrial stromal cells in pregnant rat uterus [133]. In the present study, it was confirmed by immunohistochemistry analysis that the cannabinoid receptors and AEA-metabolic enzymes are expressed in epithelial cells of lumen and glands, and to a lesser extent, in the myometrium. The epithelial localization of the ECS elements was described in a previous study [134], but within the different phases of the menstrual cycle. Present results further extend those previous ones by showing that E2-induced expression of ECS elements is co-localized within the known site of expression of ERs in the uterine tissue [135].

It is known that steroid hormones, like E2, can modulate the ECS in the CNS, down regulating CB1 receptor and also increasing AEA through down regulation of FAAH activity [136]. Present results show that, in the uterus, E2 has also the ability to modulate the expression of such elements. In fact, western blot analysis and qRT-PCR revealed that EB has the ability to increase CB1, CB2, NAPE-PLD and FAAH in the uterus and that, except for FAAH protein expression, this effect is blocked by TAM administration. These results suggest that the expression of these elements may be directly controlled by E2 through ER-α, has was observed previously in a study regarding FAAH transcription mechanism [111]. In addition, it has been described that E2 administration in OVX rats increases the expression of ER-α in the uterus 24h after the injection [137].

Taken together, these studies suggest that, the increased expression of ECS elements by E2 may be caused indirectly by the increase ER-α levels.

Present results show that ECS elements are expressed mainly in the epithelial cells of lumen and glands. The lack of ECS expression in TAM treated rats gives further support to the notion that although TAM induces volume increase, it does not promote cell proliferation nor functional activation. These results suggest that E2 but not TAM, is able to activate genes responsible for glandular proliferation and also ECS transcription and expression.

The AEA levels are essential for a proper implantation and overall pregnancy, thus, altered estrogen levels, as seen in post-menopause women using hormonal replacement therapy, may affect AEA plasmatic levels [138].

To our knowledge, this is the first study evaluating plasmatic and uterine AEA levels in response to E2 in uterus. EB treatment increased the expression of degrading and synthetizing AEA enzymes, although AEA levels were similar to the ones seen for the other treatments. Contrariwise, the plasmatic AEA quantification displayed a different outcome upon different treatments. While AEA increased with EB treatment, no differences were found with the other treatments. This observation has been described before in women in the ovulation phase of the cycle (when E2 peaks) [139]. It was also showed that administration of physiological levels of E2 to postmenopausal women resulted in an increase of plasmatic AEA, mainly due to increase of NAPE-PLD in the endothelial cells [138]. However, the direct effect of E2 alone in the plasmatic AEA was not described before. Even if the uterine levels did not change with EB treatment, other tissues and cells could contribute to the rise of AEA under EB influence. A suggested peripheral contributor for the AEA plasmatic levels may be the endothelial cells that, as previously described, may increase NAPE-PLD expression in response to E2 stimulation [138].

On the other hand, COX-2 plays a key role in decidualization and their main products, the PGs, also intervene in the uterine physiological outcome, more precisely PGE_2 and $PGF_{2\alpha}$. High levels of these two PGs result, most of the times, in miscarriage and E2 has the ability to influence PGs levels in the plasma as well as in the uterus. Immunohistochemistry analysis of COX-2 revealed a glandular and luminal expression with no visible differences between treatments. The western blot and qRT-PCR revealed that COX-2 has an increase in its transcription and expression when treated with EB. These results have not been described before in non-pregnant rat uteri. However, COX-2 was found in higher levels during estrous phase [140] which supports our observation about E2 ability to increase COX-2 expression. Moreover, PGE_2 and $PGF_{2\alpha}$ were also quantified in a local and plasmatic level. Plasmatic PGE_2 and $PGF_{2\alpha}$ quantification

showed no differences regarding both PGs in the different treatments. However, EB administration resulted in a decrease in the PGE_2 uterine levels but no changes were observed in the uterine $PGF_{2\alpha}$. Similar effects on those levels were previously observed [68]. Yet no correlation between uterine and plasmatic PGE_2 was established. Although there was an increase in COX-2 expression and transcription with EB treatment, it was observed a decrease in uterine levels of PGE_2, which seems contradictory but can be explained by a previous study that showed that E2 administration to OVX ewes resulted in a decrease of PGS transcription [69]. Thus, even with higher levels of COX-2 expression and transcription, the enzyme that synthetizes PGE_2 and $PGF_{2\alpha}$ may be down regulated, resulting in a consequent decrease in PGE_2.

This works presents evidences that estrogen may modulate ECS expression, which have increased interest in fertility and other reproductive events. Moreover, estrogen may also influence PGs which are intimately related to pregnancy and their cross-talk with eCBs is emerging as key players in reproduction. Additionally, this data add new information on how the ECS is regulated, which may be useful to improve some fertility issues and even other disorders related with the ECS.

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Chapter V

Annexes

Annexes

U.PORTO

Certifica-se que João Maia participou no IJUP'16 – *9º Encontro de Jovens Investigadores da Universidade do Porto*, que decorreu nos dias 17, 18 e 19 de fevereiro de 2016, na Fundação da Juventude – Palácio das Artes, tendo apresentado a comunicação oral com o título "Sex steroid influence on endocannabinoid system expression in the rat uterus".

Pela Comissão Organizadora

(A Vice-Reitora, Profª. Doutora Maria João Ramos)


CERTIFICATE OF PARTICIPATION
YES MEETING
YOUNG EUROPEAN SCIENTIST MEETING
The Organizing Committee of 11th YES Meeting declares that
JOÃO OLIVEIRA MAIA
has participated as a
PRESENTING STUDENT with the work
Sex steroid influence on endocannabinoid system expression in the rat uterus
João Maia1,2, Georgina Correia-da-Silva1, Natércia Teixeira1, Susana L S&2,3, Bruno Fonseca1,
1 UCIBIO, REQUIMTE, Laboratório de Bioquímica, Departamento Ciências Biológicas, Faculdade de Farmácia da Universidade do Porto, Porto, Portugal 2 Department of Anatomy, Faculty of Medicine, University of Porto, Alameda Prof. Hernâni Monteiro, 4200-319 Po
on the Eleventh YES - Young European Scientist - Meeting, from the 16th to the 18th September 2016, held at CIM - Centro de Investigação Médica, FMUP - Faculdade de Medicina da Universidade do Porto, Portugal.
Ana Vaz
President of the Organizing Committee of the 11th YES Meeting
Francisco Gonçalves
Vice-President of the Organizing Committee of the 11th YES Meeting